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(57) Abstract

The present invention relates to fusion proteins, and their uses. The present invention also relates to the genes encoding such fusion proteins. The present invention also relates to compositions comprising such fusion proteins for cleaning.

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PROTEASES AND THEIR VARIANTS HAVING PEPTIDE PROTEASE INHIBITORS FUSED TO THEM

TECHNICAL FIELD

This invention relates to novel protease/protease inhibitor fusion proteins useful in a variety of cleaning compositions, those compositions, and their methods of manufacture. These fusion proteins advantageously improve yield of the desired protease, control stoichiometry of the inhibitor/protease, produce more stable proteases, and thus more stable formulations, while providing active protease during the cleaning process.

BACKGROUND

Enzymes make up the largest class of naturally-occurring proteins. Each class of enzyme generally catalyzes (that is, accelerates a reaction without being consumed) a different kind of chemical reaction. One class of enzymes known as proteases, are known for their ability to hydrolyze (i.e., break down) a compound into two or more simpler compounds. This ability to hydrolyze proteins has been exploited by incorporating naturally-occurring and protein-engineered proteases as an additive to laundry detergent preparations. While various enzymes are used, many stains on clothes are proteinaceous and wide-specificity proteases can substantially improve removal of such stains.

These proteases are derived from biological sources, and as a result, must be separated from the biological milieu where they are found.

The amino acid sequence of a peptide or protein determines the characteristics of the peptide or protein. For example, alteration of even one amino acid may inactivate an enzyme, depending upon the location and nature of the change in the amino acid sequence. Several attempts have been made to alter the wild-type amino acid sequence of proteases to improve their properties.

One context where a protease's properties become significant is in cleaning compositions. For example, greater cleaning performance can be achieved if a cleaning composition (e.g., a laundry detergent) contains multiple enzymes, such as a protease, lipase, amylase, peroxidase, and/or cellulase. One problem with such protease-containing cleaning compositions is that the proteas s present in the compositions degrade other nzym s, as w ll as the proteases thems lves, during storage.

Protease inhibitors in cleaning compositions

The problem of enzyme degradation by proteas s in th composition has been approached in several ways, including the use of protease inhibitors in such compositions. Sometimes a reversible protease inhibitor is added during processing of the composition, or during processing of the protease, to prevent such storage-related degradation. However, those skilled in the art recognize the need to provide an inhibitor which inhibits protease during storage of the composition, but which allows the protease to be active in the cleaning environment.

Protease-containing cleaning compositions containing protease inhibitors are described, for example, in U.S. Patent 4,566,985, issued January 28, 1986, to Bruno et al. (liquid cleaning compositions containing benzamidine hydrohalide); European Patent Publication No. 376,705 published July 4, 1990 by Cardinali et al. (using lower aliphatic alcohol and a salt of a lower carboxylic acid and a surfactant system which is predominantly nonionic); European Patent Publication No. 381,262 published August 8, 1990 by Aronson et al. (using a mixture of a polyol and boron compound which are capable of reacting); WO 92/19707 published October 30, 1992 (liquid laundry detergents containing meta substituted boronic acids); U.S. Patent 4,908,150, issued March 13, 1990 to Hessel et al.; European Patent Application Serial No. 90/870212, published November 14, 1990; US Patent 5,178,789, issued January 12, 1993 to Estell (liquid detergents using an inhibitor that binds to the enzyme, for example, turkey ovomucoid as a reversible inhibitor to stabilize enzymes in the presence of subtilisin); Feder and Kochavi (FR2084751) disclose a reversible alkaline protease inhibitor said to stabilize a second enzyme in the presence of detergents; US Patent 5,039,446, issued August 13, 1991 to Estell (liquid detergents using an inhibitor having a certain dissociation constant); and Panandiker et al. (U.S. Patent 5,422,030) discloses an aromatic borate ester for stabilizing enzymes in laundry detergents.

Proteinaceous protease inhibitors

Proteinaceous protease inhibitors are typically long peptides (often over 28 amino acids), which bind to the active site of a protease and inhibit its activity. These inhibitors have been classified into several families (Families I to IX) based on primary amino acid sequence homologies (Laskowski, M., Jr., and I. Kato, "Prot in Inhibitors of Prot inases", ANN. REV. BIOCHEMISTRY, (1980) 49: 593-626). Included in these inhibitors are those commonly r ferred to as family VI inhibitors, such inhibitors include eglin and barley chymotrypsin inhibitor, and

family III inhibitors, such as *Streptomyces* subtilisin inhibitor (SSI), and plasminostreptin.

For example, Mikkelsen, WO 92/03529 (Novo Nordisk) discloses proteinaceous protease inhibitors, including SSI and barley chymotrypsin inhibitor which, in Mikkelsen's formulation, are said to stabilize lipase and cellulase to proteolysis over several days at room temperature. However, those in the art recognize, as does Mikkelsen, that many natural inhibitors have such a high affinity for the protease that the inhibited complex does not dissociate upon dilution into the washing machine. Mikkelsen discloses changing the P4 through P3' positions of family VI inhibitors which are said to lower inhibitor to proline, which is said to lower inhibitor affinity for the protease, but no data are given to support that assertion.

Halkier et al. WO 93/20175 (Novo Nordisk) discloses family VI inhibitors, using proline at the P1 and P4 positions and a variety of amino acid residues at the P2 and P3 positions. These changes are said to have lowered affinity for the protease, as a result of altering those amino acids in the inhibitor. Kd values are given for many such variants.

As a further example, Nielsen et al., (WO 93/17086) discloses changes at the P6 to P3' positions of family III inhibitors, including proline in position P1, said to lower the inhibitor's affinity for the protease. Neilsen also generally discloses the following subtilisin proteases; Novo, Carlsberg, BPN', 309, 147, and 168. No correlation of how the changes in amino acid sequence relate to inhibition is disclosed.

Manufacture of proteases

The manufacture of protease enzymes, including those useful in detergent compositions, poses its own unique problems. For example, protease production may be limited by self-proteolysis during the fermentation or purification process.

The addition of protease inhibitors to the cleaning composition, fermentation broth, or purification mixture requires the purchase and addition of excess of inhibitor, often at a point in the manufacturing process where hydrolysis of the protease has already begun.

As an example of addition at the fermentation step, German Patent Specification 2,131,451, filed by Nagase & Co., published D cemb r 30, 1971, discloses a process for the production of alkalin protease. This process is said to require the addition of 0.1 to 12% of a water soluble borate as an inhibitor.

These borates are said to enhanc the filtering activity and, accordingly, the enzyme yield. However, it is recognized that, at certain levels, the borate can actually retard the production of the enzyme.

As another example of addition at the fermentation step, Japanese patent publication J01-296987, published November 30, 1989, discloses a method comprising culturing a microorganism capable of producing protease in a culture medium containing a substance showing affinity to protease.

Finally, Joergensen et al., WO 93/13125, published July 8, 1993 by Novo Nordisk A/S discloses a process for production of a "protein susceptible to inactivation" in a fluid production medium by "continuously and reversibly protecting" said protein against inactivation during the production stage, then deprotecting the protein, and recovering the protein product. Inhibitors used are said to be SSI and eglin. The process is said to be useful for obtaining increased yields of the protein by reversibly inactivating the protein.

These processes may require addition of exogenous materials that may be expensive, ineffective, require further additional processing, and render the process difficult to control. The relevant art has not addressed the need to inhibit the protease in vivo.

More recently, the art has disclosed the desirability of producing both the inhibitor and protease in vivo, but no practical example of such a method appears in the art. There are several pitfalls associated with producing both the inhibitor and protease in vivo. For example, in instances where the expression of inhibitors is controlled by the same control elements as the protease, expression of the protease and inhibitor at same levels may fortuitously occur, however it is unpredictable. The art shows that it is recognized that, even if the process is successful on a laboratory scale, scale-up (e.g., for commercial use) is not straightforward. First, the expression control elements (since they have the same DNA sequence) may recombine, especially if the genes are plasmid-borne, thus deleting one of the genes, changing the net stoichiometry. Second, the position of one gene relative to other(s) may cause one to be expressed more readily than the other(s). Third, specific nucleotide sequences in the genes, protease or inhibitor, may "speed or slow" translation of the gene, affecting a difference in expression levels. Fourth, high level expression of two different (and possibly competing) genes may cause other difficulties: large amounts of two different transcripts ar required, with each transcript requiring its own set of positiv regulatory proteins, such as RNA polymerase and sigma

factors. Further, in many systems, each of the two proteins must be secreted at high levels through the cell's secretion apparatus. Thus, for the foregoing reasons, this approach is rather unpredictable.

Cost savings and increased yield could be realized if a natural inhibitor could be obtained in situ. If such a protease-producing organism would produce the appropriate (stoichiometric) amount of inhibitor concurrently with the protease, self-proteolysis could be reduced or eliminated.

It has surprisingly been found that the method of this invention stoichiometrically co-produces the protease and inhibitor, thus overcoming the problems posed by the art.

Objects of the Invention

It is an object of the present invention to provide improved methods for making compositions comprising protease enzymes.

It is an object of the invention to provide a method for manufacturing proteases resulting in higher yields and/or higher purity and/or easier purification and/or more stable protease preparations of compositions.

It is an object of the invention to provide methods of controlling the stoichiometry of protease:protease inhibitor in vivo as well as in the manufacturing, packaging and/or storage of the composition.

It is an object of the invention to produce a protease-producing organism which provides the appropriate (stoichiometric) amount of inhibitor concurrently with the protease, thus reducing or eliminating self-proteolysis.

An object is to also to manufacture proteases which are already stabilized in vivo by protease enzyme inhibitors, preferably as early as possible after translation.

It is a further object of the invention to provide expression systems and genes which reduce the probability of recombination or destabilization of the plasmid or expression system.

It is a further object of the invention to provide fusion proteins which avoid many potential pitfalls of inhibitor or protease being expressed at a higher rate than the other.

It is a further object of the invention to provide, in one protein, a protease and a protease inhibitor in vivo, where the protease becomes active during the cleaning process.

It is a further object of the invention to provide fusion proteins useful for liquid and solid cleaning compositions, including liquid and granular laundry

detergents and d tergent additives.

These and other objects of the present invention will be readily apparent as the detailed description proceeds.

SUMMARY

The present invention relates to fusion proteins, comprising as a first part, a protease, preferably a subtilisin, more preferably Alcalase®, Esperase®, Savinase®, Maxatase®, Maxacal®, and Maxapem 15®, BPN, BPN' and/or variants thereof; and, as a second part, a proteinaceous enzyme inhibitor, preferably SSI, eglin, turkey ovomucoid, CMTI, and/or their variants, more preferably an inhibitor which is specific to the protease part; and, as an optional third part, a peptide linker between the protease and the inhibitor.

The present invention also relates to the genes encoding such fusion proteins, cells containing such genes, and/or such fusion proteins, and methods for manufacturing such genes, fusion proteins, and/or cells containing the fusion proteins and/or genes.

The present invention also relates to compositions, preferably cleaning compositions, comprising such fusion proteins and/or their variants.

DESCRIPTION

The novel protease/inhibitor fusion protein contains two proteins of commercial interest. These fusion proteins minimize or avoid any unpredictable stoichiometry commonly associated with the dual expression of protease and inhibitor protein

This invention applies to any of a number of microbial proteases, including those from *Bacillus* species. The latter include Savinase, subtilisin BPN', Alcalase, and their derivatives, as well as any of a number of inhibitors, including SSI and other *Streptomyces* subtilisin inhibitors, eglin, barley chymotrypsin inhibitors, and variants thereof.

The invention also applies to any of a number of hosts, including both procaryotic and eucaryotic microbes, and plants; for example other *Bacillus* species, *Saccharomyces cereviseae*, *Pichia pastoris*, *Aspergillus*; and plants, such as barley, tomato, potato, soybean, alfalfa, tobacco, maize or others. Preferably, the gene expression elements are tailored to the host for maximum expression, this is well within the scope of the practice of the skilled artisan.

Definition of terms

As used herein, "cleaning process" or "cleaning" means the method or process used to clean an article, surfac or the like. Thus washing clothes in an

automatic washer is a cleaning process; diluting a cleaning composition into a bucket and cl aning a surface with a mop, sponge or cloth is a cleaning process; washing one's hands is a cleaning process; soaking an article of clothing in a sink with a cleaning composition and water is a cleaning process; placing a cleaning composition in a commode, and later flushing the toilet is a cleaning process; this list is exemplary and not exhaustive. The reader can envision many other cleaning processes.

As used herein, "stoichiometry" refers to a molar ratio. This ratio may be 1:1 (equimolar) 2:1, 3:2, 1:3 or any other ratio expressed as a molar ratio. Thus, it is contemplated that a "stoichiometric" fusion protein has a fixed "molar ratio" of inhibitor to protein, regardless of the numerical value of the ratio.

As used herein, "gene", "vector", "plasmid", "genome", or "chromosome" have their art recognized meanings. However, the skilled artisan will recognize that teaching how to use an expression system using a plasmid, etc. is sufficient to teach the skilled artisan how to use other systems whether they be genomic, plasmid-based, etc., whether they are used in procaryotes or eucaryotes, and whether the heterologous host is bacterial, fungus, plant, etc.

As used herein, "variant" means an enzyme having an amino acid sequence which differs from that of wild-type.

As used herein, "gene" means a gene coding for a particular protein or fusion protein.

As used herein the term "fusion protein" has its art-recognized meaning, that is two proteins are expressed as one amino acid chain, typically under the control of one regulatory element. For example, fusion proteins have been used for numerous applications over the last several years (Sambrook, J., E.F. Fritsch, and T. Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, 1989, Cold Spring Harbor Press). Currently, expression vectors are commercially available for using fusion technology to produce a protein of interest. (Cf., Sigma Catalog, Sigma-Aldrich Corp., Chicago, Ill. 1996).

Fusion proteins minimize or avoid any potential pitfalls of one protein being expressed at a higher rate than the other. The fusion protein allows the strain to synthesize specifically chosen molar amounts of protease and inhibitor simultaneously. For example, the fusion protein may be constructed to provide an equimolar amount of protease and inhibitor, or twice the molar amount of inhibitor compared to the protease, and the lik . Thus it is possible to maximize the protection of the protease from self-hydrolysis at the earliest possible time;

just after translation in vivo.

It is also contemplated that additional proteases and/or protease inhibitors, in addition to the fusion protein, are made by the same cell, either on the same plasmid as the fusion protein gene, a different plasmid coexisting in the cell, a plasmid while the fusion protein gene is chromosomal, or as part of the chromosome of the cell. In addition, one may produce either a protease with an inhibitor specific to a different protease, or a protease with the inhibitor specific to that protease in the fusion protein. The fusion protein may contain one or more protease parts, the same or different, and may contain one or more inhibitor parts, the same or different, as long as the fusion protein contains at least one protease part and at least one inhibitor part.

All percentages provided herein are by weight, and all measurements are made at 25°C, unless otherwise indicated. All documents cited are incorporated herein by reference in their entirety.

Proteases:

Proteases prepared by the present invention process can be of animal, plant or microorganism origin. The origin is not of particular concern: their activity is paramount. Known serine proteases are generally classified into the subtilisin family and the trypsin family, comprising, for example, chymotrypsin and elastase. The protease inhibitor(s) to be used in the manufacture of each such enzyme can be selected as appropriate for each of these type of enzyme being produced. Such proteases regardless of origin can be prepared for expression in heterologous hosts. Such hosts can be animals, plants or microorganisms; preferably they are plants or microorganisms; more preferably microorganisms.

Preferred are the known serine proteases. Particularly preferred is bacterial serine protease enzyme obtained from *Bacillus subtilis* and/or *Bacillus licheniformis*.

Non-limiting examples of such preferred proteases include Novo Industries A/S Alcalase®, Esperase®, Savinase® (Copenhagen, Denmark), GCI's Maxatase®, Maxacal®, and Maxapem 15® (protein-engineered Maxacal®) (Delft, Netherlands), and subtilisin BPN and BPN', which are commercially available from Sigma Chemical Company. Preferred proteases are also modified bacterial s rine proteases, such as thos made by Genencor International., Inc., (South San Francisco, Califomia) which are d scribed in European Patent Application 251,446, Wells et al., published January 7,1988,

and which is called herein "Proteas B"; U.S. Patent 5,030,378, Venegas, issued July 9, 1991, which refers to a modified bacterial serine proteolytic enzyme which is called "Protease A" herein; and the proteases described in WO 95/10615, published April 20, 1995 by Genencor International, hereby incorporated by reference. Preferred proteases are selected from the group consisting of Savinase®, Maxacal®, Alcalase®, BPN', Protease A and Protease B, the proteases described in WO 95/10615 (above), and mixtures thereof. These proteins and their gene sequences are shown in the art. For example, the amino acid sequence for subtilisin Carlsberg is further described by Smith, E.L., Delange, R.J Evans, W.H., Landon, M., and Markland, F.S., J. BIOL. CHEM., Vol. 243, pp. 2184-2191 (1968), incorporated herein by reference.

Thus a preferred embodiment of this invention pertain to fusion proteins comprising, as the first part, subtilisin enzymes, including those that have been modified by mutating the various nucleotide sequences that code for the enzyme, thereby modifying the amino acid sequence of the enzyme. The modified subtilisins (hereinafter, "subtilisin variants") of the present invention may have improved properties as compared to the wild-type subtilisin.

Protease Enzyme Inhibitors:

The term "peptide protease enzyme inhibitor", as used herein, means any polypeptide or protein reversible inhibitor of the proteolytic activity of the protease enzyme being capable of being fused to the protease in vivo. Typically, such an inhibitor contains more than 25 amino acids, and thus is long enough to be called a protein in its own right.

Preferably the inhibitor moiety is resistant to proteolysis by the corresponding protease.

Surprisingly, the fusion of protease inhibitors with Ki values of less than about $1X10^{-4}$ to the protease provides for improved properties of the protease portion and improved processes for manufacturing. The "coproduction" of protease and inhibitor in this manner still allows the fusion protein producing organism to produce the protease, perhaps with less toxicity to the cell. Without being bound by theory, it appears that the "coproduced" inhibitors limit the autolysis (i.e., self destruction) of the protease, and/or possibly also limit the destruction and/or other destructive influences of the protease on the organism.

Preferred inhibitors are those having Ki of l ss than about $1X10^{-4}$ M, preferably less than about $1X10^{-5}$ M, and most preferably less than about $1X10^{-6}$ M. Ki values ar determined by known procedur s. For xample, potential

inhibitors of the protease subtilisin BPN' are valuat d on the basis of their ability to inhibit the hydrolysis of succinyl-Ala-Ala-Pro-Phe-p-nitroanilid as described by DelMar for chymotrypsin, a protease similar to subtilisin BPN' [DelMar et al., ANALYTICAL BIOCHEMISTRY, (1979) 99, 316-320]. The rates, which obey Michaelis Menten conditions, are analyzed by the method of A. Goldstein (J. GEN. PHYSIOL., 27:529-580 (1944), "The Mechanism of Enzyme-Inhibitor-Substrate Reactions") to determine K_i values.

The term "reversible inhibitor", as used herein, means those protease inhibitors which are capable of being released from the protease enzyme to allow restored proteolytic activity, including but not limited to competitive, noncompetitive and uncompetitive inhibitors as described for example in detail in Mahler et al., BIOLOGICAL CHEMISTRY, Second Edition (published 1971 by Harper & Row) at pages 295-299, hereby incorporated by reference in its entirety.

Depending on the protease enzyme chosen, protease enzyme inhibitors can be selected using known methods. Existing proteases can be divided into serine, carboxy, cysteine, and metalloproteases. Within these classes, there are further divisions. For example, serine proteases include trypsin, subtilisin, chymotrypsin and "elastase type" proteases. The protease enzyme inhibitor(s) to be used in the manufacture of each such enzymes can be selected as appropriate for each enzyme being produced.

Since subtilisins and subtilisin mutants are preferred as proteases, preferred inhibitors inhibit those proteases within the preferred ranges disclosed above. Thus more preferred enzyme inhibitors include ovomucoid, eglin, SSI, and the like. These preferred inhibitors are disclosed in the following references, all incorporated herein by reference; S. Obata, S. Taguchi, I. Kumagai, and K. Miura, "Molecular Cloning and Nucleotide Sequence Determination of Gene Encoding *Streptomyces* Subtilisin Inhibitor (SSI)", J. BIOCHEMISTRY, 105: 367-371 (1989) and H. Rink, M. Liersch, P. Sieber, and F. Meyer, "A Large Fragment Approach to DNA Synthesis of a Gene for the Protease Inhibitor Eglin C from the Leech *Hirudo medicinalis* and its expression in *E. coli*", NUCLEIC ACIDS RESEARCH, 12: 6369-6387 (1984), M.W. Empie and M. Laskowski, Jr., "Thermodynamics and Kinetics of Single Residue Replacements in Avian Ovomucoid Third Domains: Effect on Inhibitor Interactions with Serine Proteinases, BIOCHEMISTRY, 21: 2274-2284 (1982).

How ver, oth r protease inhibitors ar known and can b readily made using shorter peptid s which ar known to bind to the proteases. These may be

combin d with suitably long optional link rs to provide positioning the inhibitor in the active site of the protease. If a short peptide inhibitor is used, the fusion protein may contain repeating units thereof or one or more protein peptide inhibitors, the same or different, may be present on the fusion protein.

Thus, in addition, the following inhibitors are useful in the invention: The pumpkin trypsin inhibitor CMTI I consists of 29 amino acids and is an inhibitor of trypsin and subtilisin. (J. Otlewski, T. Zbyryt, I. Krokoszynska, and T. Wilusz, "Inhibition of Serine Proteinases by Squash Inhibitors", BIOL. CHEM., Hoppe-Seyler, 371:589-594 (1990)). CMTI and other such inhibitors may be used or altered to confer protease specificity (J. Rozycki, G. Kupryszewski, K. Rolka, U. Ragnarsson, T. Zbyryt, I. Krokoszynska, and T. Wilusz, "Analogues of *Cucurbita maxima* Trypsin Inhibitor III (CMTI-III) with Elastase Inhibitory Activity", BIOL. CHEM., Hoppe-Seyler, 375:289-291 (1994)).

Optional linking peptides between the inhibitor and the protease

In addition, it is preferred that the inhibitor be attached to the protease via a hydrolyzable linking amino acid chain, separating the protease from the inhibitor.

One of ordinary skill in the art can construct the linker sequence between the protease and inhibitor to accomplish many different goals. For example, the amino acid residues could be designed to be a good substrate for proteolysis; further the sequence of the amino acids can be designed to facilitate post-translation separation of the protease and inhibitor, or to optimize the position of the inhibitor relative to the binding or active site of the protease.

It is preferred that this optional linking peptide is up to twenty amino acids in length. Preferably this linking peptide part is easily cleaved by the protease.

Method of Making-EXPRESSION

This invention uses protein fusion technology to stoichiometrically co-produce the protease and inhibitor. One set of gene expression signals is used to direct the expression of a fused gene leading to translation of a fusion protein consisting of a protease and an inhibitor.

The present invention also pertains to the genes encoding for such fusion proteins. Preferably, the fusion protein gene contains no identical genetic elements which would have the capability of homologous recombination. Unlike the art, which may desire to coproduce two proteins (proteas and inhibitor) under identical genetic controls, this invention avoids additional control elements, which provide more opportunities for differences in amount of inhibitor

and protease production and may lead to rearrang ment of the genom , plasmid or vector.

Genes encoding the protease and the inhibitor may be naturally derived or may be obtained synthetically. As an example, given but a small segment of the gene sequence, one may use PCR to locate and clone a gene for expressing the protein. In addition, methods are known to make large genes from clones of parts of them. As used herein, "small" and "large," when referring to length of DNA sequences or genes, refers to the difficulty in reliably making the sequence or gene. Thus a "small" sequence is reliably made, without errors, using standard techniques. Typically "small" genes are made using PCR: the entire gene may be copied reliably without several different steps. However, a "large" sequence or gene is not reliably made using a simple techniques, but usually requires PCR and ligation of several segments of the gene, since it cannot reliably be made using one PCR step.

A vector, plasmid, chromosome or genome may contain more than one fusion protein gene; it may also contain one or more additional protease or inhibitor genes, in other than the fusion protein region. It may contain a fusion protein and a protease, or a fusion protein and a proteinaceous inhibitor. Preferably, it contains a fusion protein and a proteinaceous inhibitor.

Mutagenesis of the intact gene, or parts of it are also known in the art. Several references describing methods of mutagenesis are readily available to the skilled artisan.

Particularly important to the invention is the regulation of the expression of the gene in the context of fusion proteins. For example, one may genetically engineer a microorganism to contain complete genes for each individual protein, including gene regulatory sequences. These gene regulatory sequences are derived from the protease and inhibitor genes of interest or from other genes.

Preferably, regulation of expression is from the same gene regulatory element to direct the expression of the entire gene. One set of gene expression signals is used to direct the expression of a fused gene leading to translation of a fusion protein consisting of a protease and an inhibitor.

The genes expressing the invention are made by known methods from known starting materials. For example, many plasmids used as starting materials are commercially available. For xample, M13 is commercially available starting material as is pUC19 and many oth rs. (Cf., Sigma Catalog, Sigma-Aldrich Corp., Chicago, Ill. 1996) In addition, handbooks for cloning and

PCR protocols ar availabl to th skilled artisan to eas the task of preparing the gene of interest.

Furthermore mutants of the proteins of interest are easily made using standard methods such as the Kunkel method of mutagenesis.

Method of Making-PREPARATION/PURIFICATION:

The method of the present invention comprises manufacturing and isolating fusion proteins comprising protease/protease inhibitors.

Preferably the inhibitor part has a Ki value of less than about $1X10^{-4}$. Preferred are methods which provide a fusion protein comprising protease inhibitor and protease. In a preferred embodiment, the preferred stoichiometry is 1:1 to about 3:1 (inhibitor : protease), preferably 3:2 to about 3:1 (inhibitor : protease), more preferably about 2:1 (inhibitor : protease).

Methods for manufacturing fusion proteins preferably use microorganisms, which produce the fusion protein. Manufacturing which involves microorganisms typically utilize a fermentation broth to grow the microorganism, followed by one or more purification steps to isolate the protease enzyme/inhibitor. The fusion protein may be harvested from the fermentation broth or the microorganism, depending upon the type of organism used, and the conditions of fermentation. Determination of which isolation methods to use is well within the purview of the skilled artisan.

Preferred methods use the fermentation of *Bacillus subtilis* and/or *Bacillus licheniformis* bacteria in a nutrient medium. Optionally, one may include protease inhibitor(s) both in the fermentation broth and thereafter use protease inhibitors (by carry over from the fermentation broth and/or by separate addition(s)) in the enzyme purification process step(s). However, this is not essential to the execution of the invention.

The methods of the present invention for manufacturing proteases preferably comprise:

(a) growing an organism which expresses a protease enzyme/inhibitor fusion protein in a fermentation broth; and

(b) purifying said protease enzyme/inhibitor by one or more steps to concentrate the protease enzyme/inhibitor produced in step (a).

More preferred processes use a fusion protein with a "molar ratio" of protease to inhibitor of at least about 1:1

Preferred methods use *Bacillus subtilis* bacteria to produce the proteas enzyme/inhibitor in the f rmentation broth of st p (a).

Also preferred is th production of the protease enzym /inhibitor in dry or concentrated liquid form prepared by the process of this invention.

Examples

The following examples are illustrative and are not intended to limit the invention since one skilled in the art would be able to substitute proteases, protease inhibitors and fusion proteins based on this disclosure, the following examples and the claims, without undue experimentation or deviation from the spirit or scope of the claims. Rather the disclosure, and the examples that follow, provide the skilled artisan with guidance as to how to make and use the invention.

Many known starting materials are used for these examples. Some of these materials are commercially available. For example, CJ236 and JM101 are known E. coli strains, pUB110 is a known plasmid and the Kunkel method of mutagenesis is also well known in the art. (See for example, Molecular Cloning, A Laboratory Manual, Second Edition, J. Sambrook, E.F. Fritsch, and T. Maniatis, Cold Spring Harbor Laboratory Press, 1989)

The fusion proteins of the invention may be made by expression systems and by various methods in various hosts, these methods are within the scope of the practice of the skilled artisan in molecular biology, biochemistry or other arts related to biotechnology.

Preparation of fusion proteins

Example 1. Construction of a Synthetic Eglin Gene

mPG296, a derivative of M13mp19 described below, is used as a cloning vector for the synthetic eglin gene. The modification of M13mp19 to mPG296 is merely a convenient starting material and it is expected that the artisan may repeat this example using M13mp19, as a starting material.

mPG296 is constructed by oligonucleotide-directed mutagenesis of M13mp19, using oligonucleotide:

5' TAA ACA ACT TTC AAC AGT CTC GAG ACT AGT TTC AGC GGA GTG AGA ATA 3' (SEQ ID NO: 1),

which places a *Spe*I and *Xho*I sites between the coding sequence for the second and third amino acid residues of mature phage protein pIII, present in mPG 296.

An glin g n is assembled by thr cycles of PCR (followed by ligation to mPG296 DNA). First, PCR is carried out with oligonucl otides of the following sequence

5' TAC TTC ACT CTG CAT TAC CCG CAG TAC GAC GTT TAC TTC CTG CCG GAA GGT TCT CCT GTT ACT CTG GAC CTG CGT TAC 3' (SEQ ID NO: 2)

and

5' CAG AGT AAC AGG AGA ACC TTC CGG CAG GAA GTA AAC GTC GTA CTG CGG GTA ATG CAG AGT GAA GTA TTC ACG AGC 3' (SEQ ID NO: 3).

Twenty-five cycles of PCR are carried out, with denaturation for one minute at 94°C, annealing for one minute at 45°C, and elongation for one minute at 72°C.

10 µl of the PCR product is used in a second 100 µl PCR containing the following two oligonucleotides:

5' AA TTC ACT GAA TTT GGT TCT GAA CTG AAA TCT TTC CCA GAA GTT GTT GGT AAA ACT GTT GAC CAG GCT CGT GAA 3' (SEQ ID NO: 4)

and

5' G ATC CTA ACC AAC ATG CGG AAC ATG GTT AAC AAC GTT AGT ACC TGG GTT GTA GAA AAC ACG AAC ACG GTT GTA ACG CAG GTC 3' (SEQ ID NO: 5).

This procedure extends the DNA to include both the 5' and 3' end of the gene.

Because these oligonucleotides do not contain full restriction sites at their termini, a third round of PCR is used to add *Eco*RI and *Bam*HI sites (thus enabling sub-cloning of the gene) with the following oligonucleotides:

5' GAT TAC GAA TTC ACT GAA TTT GGT TCT GAA 3' (SEQ ID NO: 6)

and

5' TCT AGA GGA TCC TAA CCA ACA TGC GGA 3' (SEQ ID NO: 7).

This PCR product is treated with 10 µg proteinase K, extracted twice with a phenol/chloroform mixture, and precipitated using 10 µg yeast tRNA, 300 mM sodium acetate pH 6, and 2 1/2 volumes of ethanol. This DNA is resuspended in water and digested with *Eco*RI and *Bam*HI.

This digest is extracted with phenol/chloroform, ethanol precipitated, and resuspended in water. The resulting DNA is then ligated to suitably purified *Eco*RI, *Bam*HI-digested mPG296 DNA. This ligation mix is used to transform *E. coli* JM101. Isopropylthio-β-galactoside (IPTG) and X-gal are included in the plates to distinguish blue (Lac^+) from clear (Lac^-) plaques.

Clear plaques are screened for the presence of DNA containing an insert with a *Hpa*I site. Several positive phage DNAs are found. DNA sequence analysis confirms the presence of the eglin DNA. One such phag is hereafter referred to as mPG1023.

Example 2. Alternate Construction of a Synth tic Eglin Gene

As an alternative to the approach described in Example 1, one may substitute the oligonucleotides (Seq ID NO: 4.5) in the second PCR, with the following sequences:

5' GAT TAC GAA TTC ACT GAA TTT GGT TCT GAA CTG AAA TCT TTC CCA GAA GTT GTT GGT AAA ACT GTT GAC CAG GCT CGT GAA 3' (SEQ ID NO: 8)

and

5' TCT AGA GGA TCC TAA CCA ACA TGC GGA ACA TGG TTA ACA ACG TTA GTA CCT GGG TTG TAG AAA ACA CGA ACA CGG TTG TAA CGC AGG TC 3' (SEQ ID NO: 9)

This method avoids the third round of PCR, described in Example 1. The PCR product described here is similarly purified, digested, and ligated as in Example 1, with the same recombinant phage identification.

Example 3. Construction of a Synthetic SSI Gene

The synthetic gene, encoding mature SSI, is also constructed using PCR. Because the SSI gene is larger than the eglin gene, the gene is cloned in two PCR fragments using the following oligonucleotides:

5' TCC GAC GAA TTC GAT GCT CCT TCT GCA CTT TAT GCA CCT TCA GCA TTA GTT TTA ACA GTT 3' (SEQ ID NO: 10)

5' CCT GAA AGA GCA GTA ACT CTT ACA TGT GCT CCA GGC CCT TCT GGT ACA CAT CCA GCA GCT 3' (SEQ ID NO: 11)

5' ACC TCC TAC TGC AGC TAA ATC TGC ACA TGC AGA GCC AGC TGC TGG ATG TGT 3' (SEQ ID NO: 12)

5' TAC TGC TCT TTC AGG TGC AGC TGT CGT AGC GCT AAC TCC TTT ACC AAC TGT TAA AAC TAA 3' (SEQ ID NO: 13)

5' GAT TTA GCT GCA GTA GGA GGT GAC TTA AAC GCA TTA ACA CGT GGT GAA GAC GTT ATG TGT 3' (SEQ ID NO: 14)

5' GTT GAT GGA GTT TGG CAA GGT AAA CGC GTA TCT TAT GAA CGT GTA TTT TCA AAT GAA TGT 3' (SEQ ID NO: 15)

5' TGT CCA AAG CTT GGA TCC TTA AAA TGC AAA TAC AGA AGA GCC ATG AGC GTT CAT TTC ACA TTC ATT TGA AAA 3' (SEQ ID NO: 16)

5' CCA AAC TCC ATC AAC AGT CAG TAA TAC AGG ATC ATA AAC CAT TGG ACA CAT AAC GTC TTC 3' (SEQ ID NO: 17).

The oligonucleotid s ar used pairwise in four different PCRs:

SEQ ID NO: 10 with SEQ ID NO: 13,

SEQ ID NO: 11 with SEQ ID NO: 12,
SEQ ID NO: 14 with SEQ ID NO: 17, and
SEQ ID NO: 15 with SEQ ID NO: 16.

10.6 µl of each of the first two PCR products are mixed and used in a 100 µl PCR. To obtain a sufficient quantity of DNA, eight of these PCR tubes are prepared. The PCR products of the third and fourth PCRs are similarly treated. The PCR products are treated with proteinase K, extracted with phenol/chloroform, and precipitated using ethanol.

The product of SEQ ID NO: 10-13 is digested with *Eco*RI and *Pst*I, and the product of SEQ ID NO:14-17 is digested with *Pst*I and *Hin*dIII. Following phenol/chloroform extraction and ethanol precipitation, these DNAs are ligated to pUC19 DNA which had been digested with the same restriction enzymes. The ligation mix is used to transform *E. coli* TG1, selecting on ampicillin plates containing XGAL and IPTG.

White colonies are cultured for DNA preparations. The DNA is characterized by restriction analysis and DNA sequencing. Plasmid pPG1364 contains the synthetic SSI partial gene encoded by oligonucleotides SEQ ID NO: 10-13. Plasmid pPG1366 contains the SSI partial gene encoded by oligonucleotides SEQ ID NO: 14-17.

To construct a complete synthetic gene encoding mature SSI, pPG1364 is digested with *Eco*RI, *Bgl*I, and *Pst*I. pPG1366 is digested with *Eco*RI, *Pst*I, and *Xba*I. The DNAs are ligated. Plasmid pPG1371 contains the assembled synthetic SSI gene and comprises the large *Pst*I *Eco*RI fragment of pPG1366 and the small *Eco*RI *Pst*I fragment of pPG1364. pPG1371 is used for subsequent work.

Example 4. Construction of a Subtilisin Gene-based Expression Plasmid

The expression plasmid is derivatived from pPG580, made as follows: pPG580 is a derivative of plasmids used for production of a 34 amino acid residue fragment of human parathyroid hormone (Optimization of the signal-sequence cleavage site for secretion from *Bacillus subtilis* of a 34-amino acid fragment of human parathyroid hormone, Saunders et al., GENE, 102:277-282 (1991). pPG580 is made of four parts.

First, pPG580 contains a *Bacillus amyloliquefaciens* subtilisin gene, flanked by *Hin*dIII sites, made using PCR with the following oligonucleotides.
SEQ ID NO:18 5' AGA TCC AAG CTT TTC CGC AAT TAT ATC ATT 3'
and

SEQ ID NO:19 5' GGA TTC AAG CTT TGC TCA GTT TTG CTT CTG 3'.
pPG580 contains all but the first base pair of the subtilisin gene sequence as shown by Vasantha et al. (Genes for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a large open reading frame between the regions coding for signal sequence and mature protein, J. BACTERIOLOGY, 159:811-819. 1984).

Second, adjacent to the 3' end of the subtilisin gene is a fragment of (i.e., the beginning of) pBS^+ (commercially available from Stratagene, La Jolla, CA), which extends about 3.2 kbp from the *Hin*dIII site to the pBS^+ *Xba*I site.

Third, adjacent to this *Xba*I site, a large fragment of pUB110 is inserted. (The nucleotide sequence of pUB110: some salient features in relation to replication and its regulation. McKenzie et al., PLASMID, 15: 93-103, 1986). This fragment extends 4.3 kbp from the pUB110 *Xba*I site, to its *Bam*HI site. The 324 bp fragment of pUB110, from the *Bam*HI site back to the *Xba*I I site, is thus not present in pPG580.

Fourth, the *Bam*HI site of pUB110 is no longer intact, but is fused to the *Bgl*II site of the 32 bp synthetic sequence described by Saunders et al., (Optimization of the signal-sequence cleavage site for secretion from *Bacillus subtilis* of a 34-amino acid fragment of human parathyroid hormone, Saunders et al., GENE, 102:277-282, 1991). This 32 bp sequence is shown below:
SEQ ID NO:20 5' AAG CTT CAG GAT GTT CAT AAT TTT TAA AGA TC 3'
Thus providing the plasmid, pPG580.

pPG580 is modified for further use. The *Bam*HI site and the *Eco*RI sites are removed by digestion with *Eco*RI and *Bam*HI. This digest is then incubated with T4 DNA polymerase and deoxyribonucleotide triphosphates, in the presence of ligase buffer. The resulting plasmid hereafter referred to as pPG1301, which lacks *Bam*HI or *Eco*RI restriction sites, and is used to transform *E. coli* JM101. Single-stranded DNA is prepared from a CJ236 host harboring pPG1301, referred to as strain PG1229.

pPG1301 is modified by oligonucleotide-directed mutagenesis to provide an *Eco*RI site following the signal sequence coding region and a *Bam*HI site following the translational stop coding region. The mutagenesis is carried out with the following oligonucleotides:
5' GCC CAG GCG GCA GGG GAA TTC AAA TCA AAC GGG GAA 3' (SEQ ID NO: 21)
and

5' GCG GCA GCT CAG TAA GGA TCC AAC ATA AAA AAC CGG C 3' (SEQ ID NO: 22).

The plasmid having modifications from mutagenesis by both oligomers is referred to as pPG1315. Where only SEQ ID NO. 21 mutagenized the plasmid (thus incorporating the *Bam*HI site only), the resulting plasmid is referred to hereinafter as pPG1306.

The mutagenesis is carried out using the Kunkel method (In MOLECULAR CLONING, A LABORATORY MANUAL, Second Edition, J. Sambrook, E.F. Fritsch, and T. Maniatis, Cold Spring Harbor Laboratory Press, 1989). Plasmid pPG1315 contains both the *Eco*RI and *Bam*HI sites. pPG1315 is the expression vector into which the synthetic SSI gene is inserted.

Example 5. *B. subtilis* Strain Producing Subtilisin and SSI as a fusion protein

Plasmid pPG1306 is modified by inserting a *Eco*RI restriction site is inserted after the sequence encoding the last amino acid residue of subtilisin. This modification enables fusion protein production with subtilisin. The following oligonucleotide is used in standard mutagenesis:

5' GTA CAG GCG GCA GCT CAG GAA TTC TAG TAA GGA TCC AAC ATA A 3' (SEQ ID NO: 23).

The resulting plasmid is referred to as pPG1391. An *Eco*RI, *Bam*HI SSI gene-containing fragment of pPG1371 is sub-cloned into *Eco*RI, BamHI-digested pPG1391, resulting in plasmid pPG1509.

Example 6. Gene encoding Eglin-Subtilisin fusion protein

A sub-clone of mPG1023, (a phage DNA containing the eglin gene-containing *Bam*HI *Eco*RI fragment) from Example 1 is sub-cloned between the *Bam*HI and *Eco*RI sites of pPG1315, generating plasmid pPG1317. An *Eco*RI, *Bam*HI eglin gene-containing fragment from pPG1317 is then sub-cloned into *Eco*RI, *Bam*HI-digested pPG1391, resulting in plasmid pPG1500. pPG1500 is a subtilisin/eglin gene fusion encoding both mature proteins. This plasmid is used to transform competent PG632, resulting in a bacterium expressing the fusion protein. This strain is referred to as PG1604.

The following table summarizes the plasmids and expression systems described in the examples. The list is not limiting but merely provides guidance to the artisan reading the specification.

Table 1

Summary of plasmids and Bacillus strains from xampl s

Plasmid	Description	Bacillus strain	ATCC deposit #

pPG580	subtilisin gene	PG1106	
pPG1301	subtilisin gene		
pPG1304	subtilisin gene with *Eco*RI site following the signal sequence coding region		
pPG1306	subtilisin gene with *Bam*HI site following the stop codon coding region		
pPG1315	subtilisin gene with both *Eco*RI and *Bam*HI sites		
pPG1317	eglin gene cloned adjacent to signal sequence coding region of the subtilisin gene		
pPG1364	gene segment encoding the amino portion of mature SSI		
pPG1366	gene segment encoding the carboxy portion of mature SSI		
pPG1371	gene segment encoding mature SSI		
pPG1376	SSI gene cloned adjacent to signal sequence coding region of the subtilisin gene	PG1181	69955
pPG1391	subtilisin gene with *Eco*RI site following the coding sequence for the last amino acid residue and *Bam*HI site following the termination codon coding sequence		
pPG1500	subtilisin/eglin gene fusion encoding both mature proteins	PG1198	
pPG1509	subtilisin/SSI gene fusion encoding both mature proteins*	PG1604	69954

* This plasmid in E. coli is deposited as ATCC deposit # 98025

Example 7. Fermentation and Purification of the fusion protein

Fusion protein is prepared as follows. The cells are cultured overnight in 2 x YT medium (1.6% tryptone, 1% yeast extract, 0.5% sodium chloride) containing kanamycin (50 µg/ml). The cells are pelleted, and the supernatant harvested. One ml of sample is precipitated, using trichloroacetic acid. Proteins are separated by SDS gel electrophoresis and blotted for Western blot analysis using antiserum raised to heat denatured subtilisin. The antiserum recognizes proteins larger than subtilisin in the samples from both PG1198 and PG1604. Such a result is expected for the fusion proteins. In addition, there are subtilisin-sized proteins that are recognized by the antiserum. This result suggests that the fusion protein has been subject to some proteolysis, separating the subtilisin moiety from the inhibitor moiety.

Th protein is purified by standard methods, (Cf. S. Hirono, K. T. Nakamura, Y. Iitaka, and Y. Mitsui, "Crystal Structure of the Complex of Subtilisin BPN' with its Protein Inhibitor *Streptomyces* Subtilisin Inhibitor", J.

MOL. BIOL., 131:855-869 (1979) illustrating gel filtration to purify the complex.

Example 8. Characterization of the fusion protein Inhibition

Bacillus fermentation supernatants containing the fusion protein are tested for protease activity and protease inhibitor activity.

A. SSI inhibits subtilisin BPN' and the Y217L derivative of BPN'. In the control, SSI is mixed with protease and incubated for fifteen minutes at room temperature. Protease activity is then measured using the method of DelMar, E.G., C. Largman, J.W. Brodrick and M.C. Geokas, ANAL. BIOCHEM., Vol. 99, pp. 316-320, (1979)). A 0.1 M Tris, pH 8.6, 10 mM $CaCl_2$ solution is added to bring the volume to 990 µl. Addition of 10 µl of N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (20 mg/ml) begins the reaction. The reaction rate is measured by the increase in absorbance at 410 nm.

The culture supernatants of PG1198 and PG1604 are tested for their ability to inhibit the Y217L derivative of BPN'. In neither case is there significant inhibition. The amount of inhibition, if any, is less than 10% of that seen with PG1181, which makes SSI as a single protein.

The culture supernatants of PG1198 and PG1604 are also tested for their ability to produce protease. In neither case is there significant protease activity. The amount of protease activity, if any, is 100-fold below that observed with PG1106, which produces subtilisin as a single protein.

The lack of significant inhibitor activity and protease activity is consistent with a protease/inhibitor fusion protein being made where the inhibitor moiety and the protease moiety negate each other's activity. This interpretation is reinforced by the Western blot results (above) indicating that the fusion protein is made.

B. Because it is desirable to incorporate a fusion protein into laundry products, the stability in the washing machine environment is also tested.

The stability of the fusion protein is monitored by measuring protease activity over time. If the protease and inhibitor activities are stable, the level of protease activity is constant over time. However, if the inhibitor is hydrolyzed by the protease in the fusion protein, the protease activity will rise. PG1198 and PG1604 culture supernatants are mixed with a liquid detergent composition made according to the following formula:

	WT %
C_{14-15} alkyl (ethoxy 2.25) sulfonic acid	18.0

C_{12-13} alkyl ethoxylate (9)	2.0
C_{12}-N-methylglucamide	5.0
Citric acid	4.0
Ethanol	3.5
Monoethanolamine	2.0
1,2 Propanediol	7.0
Sodium Formate	0.6
Tetraethylene pentamine ethoxylate (16)	1.18
Soil release Polymer	0.15
Silicone Suds suppresser	0.10
Brightener	0.10
Water, NaOH*** and minors	Balance to 100%

This composition constitutes 1/3 of the total sample volume. 15 µl of sample is mixed with 975 µl of 0.1 M Tris HCl, pH 8.6, 0.01 M $CaCl_2$. This dilution is incubated for thirty minutes at room temperature. After incubation, substrate is added, and the amount of protease is measured.

Example 9. Shelf stability

Within one day at 31° in the presence of a standard detergent formulation, the protease activity in PG1198 rises to 15% of the protease activity of the PG1106 control. Protease activity rises more slowly with PG1604, consistent with our observations that SSI is more stable than eglin under these conditions. However, after fourteen days at 31°, the protease activity of PG1604 is 14% of the protease activity of PG1106. Thus PG1198 and PG1604 initially contain very small amounts of protease activity. Upon incubation in the presence of detergent, the inhibitor moiety became unstable, resulting in measurable protease activity. This again reinforces the conclusion that the fusion protein is made as designed, containing protease and inhibitor moieties which negate each other's activity.

Example 10. CMTI type inhibitor fusion protein

A. Using the methods described above, the subtilisin plasmid has incorporated therein a CMTI-I (inhibitor) gene for expression of a CMTI fusion protein.

B. Mutagenesis is performed on the CMTI-I portion of the plasmid to change th P1 amino acid to valine.

Example 11. CMTIII type inhibitor fusion protein

A. Using the methods described above, subtilisin plasmid has incorporated thereto a CMTI-III (inhibitor) g ne for expression of a CMTI fusion protein.

B. Mutagenesis is performed on the CMTI-III portion of the plasmid to change the P1 amino acid to valine.

Without being bound by any theory, the data can be rationalized as follows: The fusion proteins are made, and the activity data indicate that the protease and inhibitor moieties appear to bind properly. Furthermore, these data are consistent with the protease hydrolyzing the inhibitor over time. These data are also consistent with our other results indicating that SSI is more stable than eglin in the presence of protease and detergent.

Thus it is found that the fusion protein performs similarly and act as the native protease and inhibitor, notwithstanding the fact that they are a fusion protein compared to the same protease and inhibitor when not expressed as a fusion protein.

Example 12-Other mutations

The fusion protein can contain alterations in the protease portion or the inhibitor portion of the fusion protein. For example, here SSI is the inhibitor, the following variants are made using standard methods as described above. Variants of the present invention are exemplified in the tables below.

In describing the specific mutations, the sequence of the inhibitor is given, rather than the fusion protein. This is for ease in determining the actual position of the mutation relative to the original amino acid occurring in the native inhibitor. Thus for this example, the amino acid in native wild-type is given first, the position number second, and the substituted amino acid third. Thus M73D means that the Methionine (M) which appeared as the seventy third amino acid (position 73) in the native inhibitor is replaced with Aspartic acid (D). These variations may stabilize the inhibitor and/or may optimize its Ki. The numbering corresponds to that commonly used for SSI (Obata et al, supra) and ignores the four additional amino acid residues present at the amino terminus of the SSI "wild type"-like variant and SSI variants described in this patent application.

TABLE 2

Single Mutation Variants
D83C-Variant 1
M73D-Variant 4
M73P-Variant 27

TABLE 3

Double Mutation Variants
M73D D83C-Variant 2
M73P D83C-Variant 3
M70Q D83C- Variant 5

TABLE 4

Triple Mutation Variants
M73P D83C S98A-Variant 6
M73P Y75A D83C-Variant 7
M73P D83C S98V-Variant 8
M70Q M73P D83C-Variant 9
M73P V74A D83C-Variant 10
M73P V74F D83C-Variant 11
M70Q D83C S98A-Variant 12
G47D M70Q D83C-Variant 13
G47D D83C S98A-Variant 14
G47D M73P D83C-Variant 15
G47D M73D D83C-Variant 16

Table 5

Quadruple Mutation Variants
M70Q M73P V74F D83C-Variant 17
M70Q M73P V74W D83C-Variant 18
M70Q M73P D83C S98A-Variant 19
G47D M73P V74F D83C-Variant 20
G47D M73P V74W D83C-Variant 21
G47D M73P D83C S98A-Variant 22

Table 6

Quintuple Mutation Variants
G47D M70Q M73P V74F D83C-Variant 23
G47D M70Q M73P V74W D83C-Variant 24
G47D M73P V74F D83C S98A-Variant 25
G47D M73P V74W D83C S98A -Variant 26

These useful variants provide direction to the skilled artisan who may, using these examples, and the teaching of the art, make other variants tailored to the protease of interest.

For this purpose it is also cont mplated that th skilled artisan may desire

to prepare antibodies to the fusion protein of the inv ntion. These antibodies may be prepared using known methodologies.

For example, the fusion proteins of the invention can be injected into suitable mammalian subjects such as mice, rabbits, and the like. Suitable protocols involve repeated injection of the immunogen in the presence of adjuvants according to a schedule which boosts production of antibodies in the serum. The titers of the immune serum can readily be measured using immunoassay procedures, now standard in the art, employing the invention compounds as antigens.

The antisera obtained can be used directly or monoclonal antibodies may be obtained by harvesting the peripheral blood lymphocytes or the spleen of the immunized animal and immortalizing the antibody-producing cells, followed by identifying the suitable antibody producers using standard immunoassay techniques.

The polyclonal or monoclonal preparations are then useful in monitoring expression of the invention, using standard test methodologies. Thus it is also envisioned that a kit maybe prepared using these antibodies for one in the field to use to determine expression levels and the like.

Such antibodies can also be coupled to labels such as scintigraphic labels, e.g., Tc-99 or I-131, or fluorescent labels, using standard coupling methods. The labeled antibodies can also be used in competitive assays, such as kinetic assays used to determine Ki.

V. Cleaning Compositions

In another embodiment of the present invention, an effective amount of one or more fusion proteins are included in compositions useful for cleaning a variety of surfaces in need of peptide stain removal. Such cleaning compositions include detergent compositions for cleaning hard surfaces, unlimited in form (e.g., liquid and granular); detergent compositions for cleaning fabrics, unlimited in form (e.g., granular, liquid and bar formulations); dishwashing compositions (unlimited in form); oral cleaning compositions, unlimited in form (e.g., dentifrice, toothpaste and mouthwash formulations); denture cleaning compositions, unlimited in form (e.g., liquid, tablet); and contact lens cleaning compositions, unlimited in form (e.g., liquid, tablet).

The cleaning compositions also comprise, in addition to the fusion proteins described hereinbefor , one or more cl aning composition materials compatibl with th protease inhibitor. Th term "cl aning composition

mat rial", as used herein, means any liquid, solid or gaseous material s lected for the particular type of cleaning composition desired and the form of the product (e.g., liquid, granule, bar, spray, stick, paste, gel), which materials are also compatible with the fusion protein used in the composition. The specific selection of cleaning composition materials are readily made by considering the surface material to be cleaned, the desired form of the composition for the cleaning condition during use (e.g., through the wash detergent use).

As used herein, "non-fabric cleaning compositions" include hard surface cleaning compositions, dishwashing compositions, oral cleaning compositions, denture cleaning compositions and contact lens cleaning compositions.

A. Inhibitors

The term "compatible", as used herein, means the cleaning composition materials do not reduce the proteolytic activity of the fusion protein to such an extent that the protease is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.

As used herein, "effective amount of fusion protein" refers to the quantity of fusion protein necessary to achieve the enzymatic activity necessary in the specific cleaning composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and are based on many factors, such as the particular fusion protein used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (e.g., granular, bar) composition is required, and the like. Preferably the cleaning compositions comprise from about 0.0001% to about 10% of one or more fusion proteins of the present invention, more preferably from about 0.001% to about 1%, more preferably still from about 0.01% to about 0.1%. Several examples of various cleaning compositions wherein the fusion proteins may be employed are discussed in further detail below. All parts, percentages and ratios used herein are by weight unless otherwise specified.

The fusion proteins of the present invention can be used in a variety of detergent compositions. Thus the fusion proteins can be used with various conventional ingredients to provide fully-formulated hard-surface cleaners, dishwashing compositions, fabric laundering compositions and the like. Such compositions can be in the form of liquids, granules, bars and the like. Such compositions can be formulated as modern "conc ntrated" deterg nts which contain as much as 30%-60% by weight of surfactants.

B. Surfactants

The cleaning compositions herein can optionally, and preferably, contain various anionic, nonionic, zwitterionic, etc., surfactants. Such surfactants are typically present at levels of from about 5% to about 35% of the compositions.

1. Anionic Surfactants

One type of anionic surfactant which can be utilized encompasses alkyl ester sulfonates. These are desirable because they can be made with renewable, non-petroleum resources. Preparation of the alkyl ester sulfonate surfactant component can be effected according to known methods disclosed in the technical literature. For instance, linear esters of C_8-C_{20} carboxylic acids can be sulfonated with gaseous SO_3 according to "The Journal of the American Oil Chemists Society," 52 (1975), pp. 323-329. Suitable starting materials would include natural fatty substances as derived from tallow, palm, and coconut oils, etc.

The preferred alkyl ester sulfonate surfactant, especially for laundry applications, comprises alkyl ester sulfonate surfactants. Suitable salts include metal salts such as sodium, potassium, and lithium salts, and substituted or unsubstituted ammonium salts, such as methyl-, dimethyl, trimethyl, and quaternary ammonium cations, e.g. tetramethyl-ammonium and dimethyl piperdinium, and cations derived from alkanolamines, e.g. monoethanolamine, diethanolamine, and triethanolamine. Preferably, the surfactant contains C_{10}-C_{16} alkyl, and methyl, ethyl or isopropyl. Especially preferred are the methyl ester sulfonates having a C_{14}-C_{16} alkyl.

Alkyl sulfate surfactants are another type of anionic surfactant of importance for use herein. These surfactants provide excellent overall cleaning ability when used in combination with polyhydroxy fatty acid amides (see below), good grease/oil cleaning over a wide range of temperatures, concentrations, and wash times. These surfactants, or their water soluble salts or acids are of the formula $ROSO_3M$; wherein R preferably is a C_{10}-C_{24} hydrocarbyl, preferably an alkyl or hydroxyalkyl having a C_{10}-C_{20} alkyl component, more preferably a C_{12}-C_{18} alkyl or hydroxyalkyl, and M is H or a cation, e.g., an alkali metal cation (e.g., sodium, potassium, lithium), substituted or unsubstituted ammonium cations such as methyl-, dimethyl-, and trimethyl ammonium and quaternary ammonium cations, e.g., tetramethyl-ammonium and dimethyl piperdinium, and cations deriv d from alkanolamines such as ethanolamine, diethanolamine, triethanolamine, and mixtures thereof, and the like. Typically, alkyl chains of C_{12-16} are pr ferred for lower wash t mp ratures (e.g., below

about 50°C) and C_{16-18} alkyl chains ar preferred for high r wash temperatures (e.g., above about 50°C).

Alkyl alkoxylated sulfate surfactants are another category of useful anionic surfactant. These surfactants are water soluble salts or acids typically of the formula $RO(A)_mSO_3M$ wherein R is an unsubstituted C_{10}-C_{24} alkyl or hydroxyalkyl group having a C_{10}-C_{24} alkyl component, preferably a C_{12}-C_{20} alkyl or hydroxyalkyl, more preferably C_{12}-C_{18} alkyl or hydroxyalkyl, A is an ethoxy or propoxy unit, m is greater than zero, typically between about 0.5 and about 6, more preferably between about 0.5 and about 3, and M is H or a cation which can be, for example, a metal cation (e.g., sodium, potassium, lithium, calcium, magnesium, etc.), ammonium or substituted-ammonium cation. Alkyl ethoxylated sulfates as well as alkyl propoxylated sulfates are contemplated herein. Specific examples of substituted ammonium cations include methyl-, dimethyl-, trimethyl-ammonium and quaternary ammonium cations, such as tetramethyl-ammonium, dimethyl piperidinium and cations derived from alkanolamines, e.g. monoethanolamine, diethanolamine, and triethanolamine, and mixtures thereof. Exemplary surfactants are C_{12}-C_{18} alkyl polyethoxylate (1.0) sulfate, C_{12}-C_{18} alkyl polyethoxylate (2.25) sulfate, C_{12}-C_{18} alkyl polyethoxylate (3.0) sulfate, and C_{12}-C_{18} alkyl polyethoxylate (4.0) sulfate wherein M is conveniently selected from sodium and potassium.

Other anionic surfactants useful for purposes can also be included in the compositions hereof. These can include salts (including, for example, sodium, potassium, ammonium, and substituted ammonium salts such as mono-, di- and triethanolamine salts) of soap, C_9-C_{20} linear alkylbenzenesulphonates, C_8-C_{22} primary or secondary alkanesulphonates, C_8-C_{24} olefinsulphonates, sulphonated polycarboxylic acids prepared by sulphonation of the pyrolyzed product of alkaline earth metal citrates, e.g., as described in British patent specification No. 1,082,179, alkyl glycerol sulfonates, fatty acyl glycerol sulfonates, fatty oleyl glycerol sulfates, alkyl phenol ethylene oxide ether sulfates, paraffin sulfonates, alkyl phosphates, isothionates such as the acyl isothionates, N-acyl taurates, fatty acid amides of methyl tauride, alkyl succinamates and sulfosuccinates, monoesters of sulfosuccinate (especially saturated and unsaturated C_{12}-C_{18} monoesters) diesters of sulfosuccinate (especially saturated and unsaturated C_6-C_{14} diesters), N-acyl sarcosinates, sulfates of alkylpolysaccharides such as th sulfates of alkylpolyglucosid (the nonionic nonsulfat d compounds b ing describ d below), branch d primary

alkyl sulfat s, alkyl polyethoxy carboxylat s such as thos of the formula $RO(CH_2CH_2O)_kCH_2COO\text{-}M^+$ wherein R is a C_8-C_{22} alkyl, k is an integer from 0 to 10, and M is a soluble salt-forming cation, and fatty acids esterified with isethionic acid and neutralized with sodium hydroxide. Resin acids and hydrogenated resin acids are also suitable, such as rosin, hydrogenated rosin, and resin acids and hydrogenated resin acids present in or derived from tall oil. Further examples are given in SURFACE ACTIVE AGENTS AND DETERGENTS (Vol. I and II by Schwartz, Perry and Berch). A variety of such surfactants are also generally disclosed in U.S. Patent 3,929,678, issued December 30, 1975 to Laughlin, et al. at Column 23, line 58 through Column 29, line 23 (herein incorporated by reference).

2. Nonionic Detergent Surfactants

Suitable nonionic detergent surfactants are generally disclosed in U.S. Patent 3,929,678, Laughlin et al., issued December 30, 1975, at column 13, line 14 through column 16, line 6, incorporated herein by reference. Exemplary, non-limiting classes of useful nonionic surfactants are listed below.

1. The polyethylene, polypropylene, and polybutylene oxide condensates of alkyl-phenols. In general, the polyethylene oxide condensates are preferred. These compounds include the condensation products of alkyl phenols having an alkyl group containing from about 6 to about 12 carbon atoms in either a straight chain or branched chain configuration with the alkylene oxide. In a preferred embodiment, the ethylene oxide is present in an amount equal to from about 5 to about 25 moles of ethylene oxide per mole of alkyl phenol. Commercially available nonionic surfactants of this type include Igepal^R CO-630, marketed by the GAF Corporation; and Triton^R X-45, X-114, X-100, and X-102, all marketed by the Rohm & Haas Company. These compounds are commonly referred to as alkyl phenol alkoxylates, (e.g., alkyl phenol ethoxylates).

2. The condensation products of aliphatic alcohols with from about 1 to about 25 moles of ethylene oxide. The alkyl chain of the aliphatic alcohol can either be straight or branched, primary or secondary, and generally contains from about 8 to about 22 carbon atoms. Particularly preferred are the condensation products of alcohols having an alkyl group containing from about 10 to about 20 carbon atoms with from about 2 to about 18 moles of ethylene oxide p r m l of alcohol. Examples of commercially availabl nonionic surfactants of this type include Tergitol^R 15-S-9 (the condensation product of C_{11}-C_{15} linear secondary alcohol with 9 moles ethyl ne oxide), Tergitol^R 24-L-

6 NMW (the condensation product of C_{12}-C_{14} primary alcohol with 6 moles ethylene oxide with a narrow molecular weight distribution), both marketed by Union Carbide Corporation; Neodol^R 45-9 (the condensation product of C_{14}-C_{15} linear alcohol with 9 moles of ethylene oxide), Neodol^R 23-6.5 (the condensation product of C_{12}-C_{13} linear alcohol with 6.5 moles of ethylene oxide), Neodol^R 45-7 (the condensation product of C_{14}-C_{15} linear alcohol with 7 moles of ethylene oxide), Neodol^R 45-4 (the condensation product of C_{14}-C_{15} linear alcohol with 4 moles of ethylene oxide), marketed by Shell Chemical Company, and Kyro^R EOB (the condensation product of C_{13}-C_{15} alcohol with 9 moles ethylene oxide), marketed by The Procter & Gamble Company. This category of nonionic surfactant is referred to generally as "alkyl ethoxylates."

3. The condensation products of ethylene oxide with a hydrophobic base formed by the condensation of propylene oxide with propylene glycol. The hydrophobic portion of these compounds preferably has a molecular weight of from about 1500 to about 1800 and exhibits water insolubility. The addition of polyoxyethylene moieties to this hydrophobic portion tends to increase the water solubility of the molecule as a whole, and the liquid character of the product is retained up to the point where the polyoxyethylene content is about 50% of the total weight of the condensation product, which corresponds to condensation with up to about 40 moles of ethylene oxide. Examples of compounds of this type include certain of the commercially-available Pluronic^R surfactants, marketed by BASF.

4. The condensation products of ethylene oxide with the product resulting from the reaction of propylene oxide and ethylenediamine. The hydrophobic moiety of these products consists of the reaction product of ethylenediamine and excess propylene oxide, and generally has a molecular weight of from about 2500 to about 3000. This hydrophobic moiety is condensed with ethylene oxide to the extent that the condensation product contains from about 40% to about 80% by weight of polyoxyethylene and has a molecular weight of from about 5,000 to about 11,000. Examples of this type of nonionic surfactant include certain of the commercially available Tetronic^R compounds, marketed by BASF.

5. Semi-polar nonionic surfactants are a special category of nonionic surfactants which include wat r-solubl amin oxides containing on alkyl moi ty of from about 10 to about 18 carbon atoms and 2 moieties sel cted from the group consisting of alkyl groups and hydroxyalkyl groups containing from

about 1 to about 3 carbon atoms; water-soluble phosphine oxides containing one alkyl moi ty of from about 10 to about 18 carbon atoms and 2 moieties selected from the group consisting of alkyl groups and hydroxyalkyl groups containing from about 1 to about 3 carbon atoms; and water-soluble sulfoxides containing one alkyl moiety of from about 10 to about 18 carbon atoms and a moiety selected from the group consisting of alkyl and hydroxyalkyl moieties of from about 1 to about 3 carbon atoms.

Semi-polar nonionic detergent surfactants include the amine oxide surfactants, having alkyl, hydroxyalkyl, or alkyl phenyl group or mixtures thereof containing from about 8 to about 26 carbon atoms; hydroxyalkylene group or a polyethylene oxide group containing from about 1 to about 3 ethylene oxide groups. Such groups can be attached to each other, e.g., through an oxygen or nitrogen atom, to form a ring structure.

These amine oxide surfactants in particular include C_{10}-C_{18} alkyl dimethyl amine oxides and C_8-C_{12} alkoxy ethyl dihydroxy ethyl amine oxides.

6. Alkylpolysaccharides disclosed in U.S. Patent 4,565,647, Llenado, issued January 21, 1986, having a hydrophobic group containing from about 6 to about 30 carbon atoms, preferably from about 10 to about 16 carbon atoms and a polysaccharide, e.g., a polyglycoside, hydrophilic group containing from about 1.3 to about 10, preferably from about 1.3 to about 3, most preferably from about 1.3 to about 2.7 saccharide units. Any reducing saccharide containing 5 or 6 carbon atoms can be used, e.g., glucose, galactose and galactosyl moieties can be substituted for the glucosyl moieties. (Optionally the hydrophobic group is attached at the 2-, 3-, 4-, etc. positions thus giving a glucose or galactose as opposed to a glucoside or galactoside.) The intersaccharide bonds can be, e.g., between the one position of the additional saccharide units and the 2-, 3-, 4-, and/or 6- positions on the preceding saccharide units.

Optionally, and less desirably, there can be a polyalkylene-oxide chain joining the hydrophobic moiety and the polysaccharide moiety. The preferred alkyleneoxide is ethylene oxide. Typical hydrophobic groups include alkyl groups, either saturated or unsaturated, branched or unbranched containing from about 8 to about 18, preferably from about 10 to about 16, carbon atoms. Preferably, the alkyl group is a straight chain saturated alkyl group. The alkyl group can contain up to about 3 hydroxy groups and/or th polyalkyl neoxid chain can contain up to about 10, preferably less than 5, alkyleneoxide moieties.

Suitable alkyl polysaccharides are octyl, nonyl, d cyl, und cyldodecyl, tridecyl, tetradecyl, pentadecyl, hexadecyl, heptadecyl, and octadecyl, di-, tri-, tetra-, penta-, and hexaglucosides, galactosides, lactosides, glucoses, fructosides, fructoses and/or galactoses. Suitable mixtures include coconut alkyl, di-, tri-, tetra-, and pentaglucosides and tallow alkyl tetra-, penta-, and hexa-glucosides.

The preferred alkylpolyglycosides contain a capping group selected from consisting of alkyl, alkyl-phenyl, hydroxyalkyl, hydroxyalkylphenyl, and mixtures thereof in which the alkyl groups contain from about 10 to about 18, preferably from about 12 to about 14, carbon atoms; n is 2 or 3, preferably 2; t is from 0 to about 10, preferably 0; and x is from about 1.3 to about 10, preferably from about 1.3 to about 3, most preferably from about 1.3 to about 2.7. The glycosyl is preferably derived from glucose. To prepare these compounds, the alcohol or alkylpolyethoxy alcohol is formed first and then reacted with glucose, or a source of glucose, to form the glucoside (attachment at the 1-position). The additional glycosyl units can then be attached between their 1-position and the preceding glycosyl units 2-, 3-, 4- and/or 6-position, preferably predominantly the 2-position.

7. Fatty acid amide surfactants preferably having an alkyl group containing from about 7 to about 21 (preferably from about 9 to about 17) carbon atoms. Preferred amides are C_8-C_{20} ammonia amides, monoethanolamides, diethanolamides, and isopropanolamides.

3. Cationic Surfactants

Cationic surfactants can also be included in detergent compositions of the present invention. Cationic surfactants include the ammonium surfactants such as alkyldimethyl-ammonium halogenides, and those surfactants having alkyl, alkylaryl, or aryl sidechains; hydroylated or oxygenated sidechains, including polymers of cellulose of other sugars or sugar like moieties, preferably having a molecular weight less than about 1000; and X is any compatible anion.

Other cationic surfactants useful herein are also described in U.S. Patent 4,228,044, Cambre, issued October 14, 1980, incorporated herein by reference.

4. Other Surfactants

Ampholytic surfactants can be incorporated into the detergent compositions hereof. These surfactants can be broadly described as aliphatic derivatives of secondary or tertiary amines, or aliphatic derivatives of heterocyclic secondary and tertiary amin s in which the aliphatic radical can b straight chain or branched. One of the aliphatic substituents contains at least

about 8 carbon atoms, typically from about 8 to about 18 carbon atoms, and at least one contains an anionic water-solubilizing group, e.g., carboxy, sulfonate, sulfate. See U.S. Patent No. 3,929,678 to Laughlin et al., issued December 30, 1975 at column 19, lines 18-35 (herein incorporated by reference) for examples of ampholytic surfactants.

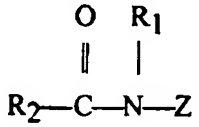
Zwitterionic surfactants can also be incorporated into the detergent compositions hereof. These surfactants can be broadly described as derivatives of secondary and tertiary amines, derivatives of heterocyclic secondary and tertiary amines, or derivatives of quaternary ammonium, quaternary phosphonium or tertiary sulfonium compounds. See U.S. Patent No. 3,929,678 to Laughlin et al., issued December 30, 1975 at column 19, line 38 through column 22, line 48 (herein incorporated by reference) for examples of zwitterionic surfactants.

Ampholytic and zwitterionic surfactants are generally used in combination with one or more anionic and/or nonionic surfactants.

The liquid detergent compositions hereof may also contain an "enzyme performance-enhancing amount" of polyhydroxy fatty acid amide surfactant. By "enzyme-enhancing" is meant that the formulator of the composition can select an amount of polyhydroxy fatty acid amide to be incorporated into the compositions that will improve enzyme cleaning performance of the detergent composition. In general, for conventional levels of enzyme, the incorporation of about 1%, by weight, polyhydroxy fatty acid amide will enhance enzyme performance.

The detergent compositions hereof will typically comprise at least about 1% weight basis, polyhydroxy fatty acid amide surfactant and preferably at least from about 3% to about 50%, most preferably from about 3% to 30%, of the polyhydroxy fatty acid amide.

The polyhydroxy fatty acid amide surfactant is of formula



(I)

where R^1 is H, C_1-C_4 hydrocarbyl, 2-hydroxy ethyl, 2-hydroxy propyl, or a mixture thereof, preferably C_1-C_4 alkyl, more preferably C_1 or C_2 alkyl, most preferably C_1 alkyl (i.e., methyl); and R^2 is a C_5-C_{31} hydrocarbyl, pref rably straight chain C_7-C_{19} alkyl or alkenyl, more pr f rably straight chain C_9-C_{17} alkyl or alkenyl, most preferably straight chain C_{11}-C_{15} alkyl or alkenyl, or

mixtures thereof; and Z is a polyhydroxyhydrocarbyl having a linear hydrocarbyl chain with at least 3 hydroxyls directly connected to the chain, or an alkoxylated derivative (preferably ethoxylated or propoxylated) thereof. Z preferably will be derived from a reducing sugar in a reductive amination reaction; more preferably Z will be a glycityl. Suitable reducing sugars include glucose, fructose, maltose, lactose, galactose, mannose, and xylose. As raw materials, high dextrose corn syrup, high fructose corn syrup, and high maltose corn syrup can be utilized as well as the individual sugars listed above. These corn syrups may yield a mix of sugar components for Z. It should be understood that it is by no means intended to exclude other suitable raw materials. Z preferably will be selected from the group consisting of $-CH_2-(CHOH)_n-CH_2OH$, $-CH(CH_2OH)-(CHOH)_{n-1}-CH_2OH$,
$-CH_2-(CHOH)_2(CHOR')(CHOH)-CH_2OH$, and alkoxylated derivatives thereof, where n is an integer from 3 to 5, inclusive, and R' is H or a cyclic or aliphatic monosaccharide. Most preferred are glycityls wherein n is 4, particularly $-CH_2-(CHOH)_4-CH_2OH$.

In Formula (I), R' can be, for example, N-methyl, N-ethyl, N-propyl, N-isopropyl, N-butyl, N-2-hydroxy ethyl, or N-2-hydroxy propyl.

R2-CO-N< can be, for example, cocamide, stearamide, oleamide, lauramide, myristamide, capricamide, palmitamide, tallowamide, etc.

Z can be 1-deoxyglucityl, 2-deoxyfructityl, 1-deoxymaltityl, 1-deoxylactityl, 1-deoxygalactityl, 1-deoxymannityl, 1-deoxymaltotriotityl, etc.

Methods for making polyhydroxy fatty acid amides are known in the art. In general, they can be made by reacting an alkyl amine with a reducing sugar in a reductive amination reaction to form a corresponding N-alkyl polyhydroxyamine, and then reacting the N-alkyl polyhydroxyamine with a fatty aliphatic ester or triglyceride in a condensation/amidation step to form the N-alkyl, N-polyhydroxy fatty acid amide product. Processes for making compositions containing polyhydroxy fatty acid amides are disclosed, for example, in G.B. Patent Specification 809,060, published February 18, 1959, by Thomas Hedley & Co., Ltd., U.S. Patent 2,965,576, issued December 20, 1960 to E. R. Wilson, and U.S. Patent 2,703,798, Anthony M. Schwartz, issued March 8, 1955, and U.S. Patent 1,985,424, issued December 25, 1934 to Piggott, each of which is incorporated herein by r fer nce.

Nonlimiting examples of surfactants us ful h rein includ th $C_{11}-C_{18}$ alkyl b nz n sulfonates and primary and random alkyl sulfates, the $C_{10}-C_{18}$

s condary (2,3) alkyl sulfates of the formulas $CH_3(CH_2)x(CHOSO_3)^-M^+)CH_3$ and $CH_3(CH_2)y(CHOSO_3^-M^+)$ CH_2CH_3 wherein x and (y+1) are integers of at least about 7, preferably at least about 9, and M is a water-solubilizing cation, especially sodium, the C_{10}-C_{18} alkyl alkoxy sulfates (especially EO 1-5 ethoxy sulfates), C_{10}-C_{18} alkyl alkoxy carboxylates (especially the EO 1-5 ethoxycarboxylates), the C_{10}-C_{18} alkyl polyglycosides, and their corresponding sulfated polyglycosides, C_{12}-C_{18} alpha-sulfonated fatty acid esters, C_{12}-C_{18} alkyl and alkyl phenol alkoxylates (especially ethoxylates and mixed ethoxy/propoxy), C_{12}-C_{18} betaines and sulfobetaines ("sultaines"), C_{10}-C_{18} amine oxides, and the like. The alkyl alkoxy sulfates (AES) and alkyl alkoxy carboxylates (AEC) are preferred herein. (Use of such surfactants in combination with the aforesaid amine oxide and/or betaine or sultaine surfactants is also preferred, depending on the desires of the formulator.) Other conventional useful surfactants are listed in standard texts. Particularly useful surfactants include the C_{10}-C_{18} N-methyl glucamides disclosed in US Patent 5, 194,639, Connor et al., issued March 16, 1993, incorporated herein by reference.

A wide variety of other ingredients useful in detergent cleaning compositions can be included in the compositions herein, including other active ingredients, carriers, hydrotropes, processing aids, dyes or pigments, solvents for liquid formulations, etc. If an additional increment of sudsing is desired, suds boosters such as the C_{10}-C_{16} alkolamides can be incorporated into the compositions, typically at about 1% to about 10% levels. The C_{10}-C_{14} monoethanol and diethanol amides illustrate a typical class of such suds boosters. Use of such suds boosters with high sudsing adjunct surfactants such as the amine oxides, betaines and sultaines noted above is also advantageous. If desired, soluble magnesium salts such as $MgCl_2$, $MgSO_4$, and the like, can be added at levels of, typically, from about 0.1% to about 2%, to provide additionally sudsing.

C. Proteases and other enzymes

The formulator may wish to employ various additional enzymes, such as cellulases, lipases, amylases and proteases in such compositions, typically at levels of from about 0.001% to about 1% by weight. Various fabric care enzymes are well-known in the laundry deterg nt art.

Preferred compositions herein further comprise a performance-enhancing amount of a detergent-compatible second enzyme. By "detergent-compatible" is

meant compatibility with the oth r ingredi nts of a liquid deterg nt composition, such as surfactant and detergency builder. These second enzymes are preferably selected from the group consisting of lipase, amylase, cellulase, and mixtures thereof. The term "second enzyme" excludes the proteases discussed above, so each composition contains at least two kinds of enzyme, including at least one protease. The amount of second enzyme used in the composition varies according to the type of enzyme. In general, from about 0.0001 to 0.3, more preferably 0.001 to 0.1, weight % of these second enzymes are preferably used. Mixtures of the same class of enzymes (e.g. lipase) or two or more classes (e.g. cellulase and lipase) may be used. Purified or non-purified forms of the enzyme may be used.

Any lipolytic enzyme suitable for use in a liquid detergent composition can be used in these compositions. Suitable lipase enzymes for use herein include those of bacterial and fungal origin.

Suitable bacterial lipases include those produced by microorganisms of the Pseudomonas groups, such as Pseudomonas stutzeri ATCC 19.154, as disclosed in British Patent 1,372,034, incorporated herein by reference. Suitable lipases include those which show a positive immunological cross-reaction with the antibody of the lipase produced by the microorganism Pseudomonas fluorescens IAM 1057. This lipase and a method for its purification have been described in Japanese Patent Application 53-20487, laid open on February 24, 1978. This lipase is available from Amano Pharmaceutical Co. Ltd., Nagoya, Japan, under the trade name Lipase P "Amano," hereinafter referred to as "Amano-P." Such lipases should show a positive immunological cross-reaction with the Amano-P antibody, using the standard and well-known immunodiffusion procedure according to Ouchterlony (Acta. Med. Scan., 133, pages 76-79 (1950)). These lipases, and a method for their immunological cross-reaction with Amano-P, are also described in U.S. Patent 4,707,291, Thom et al., issued November 17, 1987, incorporated herein by reference. Typical examples thereof are the Amano-P lipase, the lipase ex Pseudomonas fragi FERM P 1339 (available under the trade name Amano-B), lipase ex Pseudomonas nitroreducens var. lipolyticum FERM P 1338 (available under the trade name Amano-CES), lipases ex Chromobacter viscosum, e.g. Chromobacter viscosum var. lipolyticum NRRLB 3673, commercially availabl from Toyo Jozo Co., Tagata, Japan; and further Chromobacter viscosum lipas s from U.S. Bioch mical Corp., U.S.A. and Disoynth Co., The Netherlands, and

lipases ex Pseudomonas gladioli.

Suitable fungal lipases include those producible by Humicola lanuginosa and Thermomyces lanuginosus. Most preferred is lipase obtained by cloning the gene from Humicola lanuginosa and expressing the gene in Aspergillus oryzae as described in European Patent Application 0 258 068 (Novo Industries A/S), commercially available from Novo Nordisk A/S under the trade name Lipolase^R.

From about 10 to 18000, preferably about 60 to 6000, lipase units per gram (LU/g) of lipase can be used in these compositions. A lipase unit is that amount of lipase which produces 1 mmol of titratable fatty acid per minute in a pH stat, where pH is 9.0, temperature is 30°C, substrate is an emulsion of 3.3wt % of olive oil and 3.3% gum arabic, in the presence of 13 mmol/l Ca^{++} and 20 mmol/l NaCl in 5 mmol/l Tris-buffer.

Any cellulase suitable for use in a liquid detergent composition can be used in these compositions. Suitable cellulase enzymes for use herein include those from bacterial and fungal origins. Preferably, they will have a pH optimum of between 5 and 9.5. From about 0.0001 to 0.1 weight % cellulase can be used.

Suitable cellulases are disclosed in U.S. Patent 4,435,307, Barbesgaard et al., issued March 6, 1984, incorporated herein by reference, which discloses fungal cellulase produced from Humicola insolens. Suitable cellulases are also disclosed in GB-A-2.075.028, GB-A-2.095.275 and DE-OS-2.247.832.

Examples of such cellulases are cellulases produced by a strain of Humicola insolens (Humicola grisea var. thermoidea), particularly the Humicola strain DSM 1800, and cellulases produced by a fungus of Bacillus N or a cellulase 212-producing fungus belonging to the genus Aeromonas, and cellulase extracted from the hepatopancreas of a marine mollusc (Dolabella Auricula Solander).

Any amylase suitable for use in a liquid detergent composition can be used in these compositions. Amylases include, for example, amylases obtained from a special strain of B.licheniformis, described in more detail in British Patent Specification No. 1,296,839 (Novo). Amylolytic proteins include, for example, Rapidase^R, International Bio-Synthetics, Inc. and Termamyl^R Novo Industries.

From about 0.0001% to 0.55, preferably 0.0005 to 0.1, wt. % amylase can be used.

D. Other (Optional) Ingr dients

The liquid detergent compositions herein can contain water and other solvents as carriers. Low molecular weight primary or secondary alcohols exemplified by methanol, ethanol, propanol, and isopropanol are suitable. Monohydric alcohols are preferred for solubilizing surfactants, but polyols such as those containing from about 2 to about 6 carbon atoms and from about 2 to about 6 hydroxy groups (e.g., 1,3-propanediol, ethylene glycol, glycerine, and 1,2-propanediol) can also be used. The compositions may contain from about 5% to about 90%, typically from about 10% to about 50% of such carriers.

The detergent compositions herein will preferably be formulated such that during use in aqueous cleaning operations, the wash water will have a pH between about 6.8 and about 11.0. Finished products thus are typically formulated at this range. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

Various bleaching compounds, such as the percarbonates, perborates and the like, can be used in such compositions, typically at levels from about 1% to about 15% by weight. If desired, such compositions can also contain bleach activators such as tetraacetyl ethylenediamine, nonanoyloxybenzene sulfonate, and the like, which are also known in the art. Usage levels typically range from about 1% to about 10% by weight.

Various soil release agents, especially of the anionic oligoester type, various chelating agents, especially the aminophosphonates and ethylenediaminedisuccinates, various clay soil removal agents, especially ethoxylated tetraethylene pentamine, various dispersing agents, especially polyacrylates and polyasparatates, various brighteners, especially anionic brighteners, various suds suppressors, especially silicones and secondary alcohols, various fabric softeners, especially smectite clays, and the like can all be used in such compositions at levels ranging from about 1% to about 35% by weight. Standard formularies and published patents contain multiple, detailed descriptions of such conventional materials.

Enzyme stabilizers may also be used in the cleaning compositions. Such stabilizers include propylene glycol (preferably from about 1% to about 10%), sodium formate (preferably from about 0.1% to about 1%) and calcium formate (pr f rably from about 0.1% to about 1%).

When formulating the hard surface cleaning compositions and fabric cleaning compositions of the pr s nt inv ntion, the formulator may wish to

employ various builders at levels from about 5% to about 50% by weight. Typical builders include the 1-10 micron zeolites, polycarboxylates such as citrate and oxydisuccinates, layered silicates, phosphates, and the like. Other conventional builders are listed in standard formularies.

From 0 to about 50 weight % detergency builder can be used herein. Inorganic as well as organic builders can be used. When present, the compositions will typically comprise at least about 1% builder. Liquid formulations preferably comprise from about 3% to 30%, more preferably about 5 to 20%, by weight, of detergent builder.

Inorganic detergent builders include, but are not limited to, the alkali metal, ammonium and alkanolammonium salts of polyphosphates (exemplified by the tripolyphosphates, pyrophosphates, and glassy polymeric meta-phosphates), phosphonates, phytic acid, silicates, carbonates (including bicarbonates and sesquicarbonates), sulphates, and aluminosilicates. Borate builders, as well as builders containing borate-forming materials that can produce borate under detergent storage or wash conditions (hereinafter, collectively "borate builders"), can also be used. Preferably, non-borate builders are used in the compositions of the invention intended for use at wash conditions less than about 50°C, especially less than about 40°C.

Examples of silicate builders are the alkali metal silicates, particularly those having a SiO_2:Na_2O ratio in the range 1.6:1 to 3.2:1 and layered silicates, such as the layered sodium silicates described in U.S. Patent 4,664,839, issued May 12, 1987 to H. P. Rieck, incorporated herein by reference. However, other silicates may also be useful such as for example magnesium silicate, which can serve as a crispening agent in granular formulations, as a stabilizing agent for oxygen bleaches, and as a component of suds control systems.

Examples of carbonate builders are the alkaline earth and alkali metal carbonates, including sodium carbonate and sesquicarbonate and mixtures thereof with ultra-fine calcium carbonate as disclosed in German Patent Application No. 2,321,001 published on November 15, 1973, the disclosure of which is incorporated herein by reference.

Aluminosilicate builders are useful in the present invention. Aluminosilicate builders are of great importance in most currently marketed heavy duty granular detergent compositions, and can also be a significant build r ingredient in liquid detergent formulations. Aluminosilicate build rs includ thos having the mpirical formula:

$$M_z(zAlO_2 \cdot ySiO_2)$$

wherein M is sodium, potassium, ammonium or substituted ammonium, z is from about 0.5 to about 2; and y is 1; this material having a magnesium ion exchange capacity of at least about 50 milligram equivalents of $CaCO_3$ hardness per gram of anhydrous aluminosilicate. Preferred alumino-silicates are zeolite builders which have the formula:

$$Na_z[(AlO_2)_z (SiO_2)_y] \cdot xH_2O$$

wherein z and y are integers of at least 6, the molar ratio of z to y is in the range from 1.0 to about 0.5, and x is an integer from about 15 to about 264.

Useful aluminosilicate ion exchange materials are commercially available. These aluminosilicates can be crystalline or amorphous in structure and can be naturally-occurring aluminosilicates or synthetically derived. A method for producing aluminosilicate ion exchange materials is disclosed in U.S. Patent 3,985,669, Krummel, et al., issued October 12, 1976, incorporated herein by reference. Preferred synthetic crystalline aluminosilicate ion exchange materials useful herein are available under the designations Zeolite A, Zeolite P (B), and Zeolite X. In an especially preferred embodiment, the crystalline aluminosilicate ion exchange material has the formula:

$$Na_{12}[(AlO_2)_{12}(SiO_2)_{12}] \cdot xH_2O$$

wherein x is from about 20 to about 30, especially about 27. This material is known as Zeolite A. Preferably, the aluminosilicate has a particle size of about 0.1-10 microns in diameter.

Specific examples of polyphosphates are the alkali metal tripolyphosphates, sodium, potassium and ammonium pyrophosphate, sodium and potassium and ammonium pyrophosphate, sodium and potassium orthophosphate, sodium polymeta phosphate in which the degree of polymerization ranges from about 6 to about 21, and salts of phytic acid.

Examples of phosphonate builder salts are the water-soluble salts of ethane 1-hydroxy-1, 1-diphosphonate particularly the sodium and potassium salts, the water-soluble salts of methylene diphosphonic acid e.g. the trisodium and tripotassium salts and the water-soluble salts of substituted methylene diphosphonic acids, such as the trisodium and tripotassium ethylidene, isopyropylidene benzylmethylidene and halo methylidene phosphonates. Phosphonat builder salts of the aforem ntioned types are disclos d in U.S. Pat nt Nos. 3,159,581 and 3,213,030 issued Dec mber 1, 1964 and October 19, 1965, to Diehl; U.S. Pat nt No. 3,422,021 issued January 14, 1969, to Roy;

and U.S. Patent Nos. 3,400,148 and 3,422,137 issued September 3, 1968, and January 14, 1969 to Quimby, said disclosures being incorporated herein by reference.

Organic detergent builders preferred for the purposes of the present invention include a wide variety of polycarboxylate compounds. As used herein, "polycarboxylate" refers to compounds having a plurality of carboxylate groups, preferably at least 3 carboxylates.

Polycarboxylate builder can generally be added to the composition in acid form, but can also be added in the form of a neutralized salt. When utilized in salt form, alkali metals, such as sodium, potassium, and lithium, or alkanolammonium salts are preferred.

Included among the polycarboxylate builders are a variety of categories of useful materials. One important category of polycarboxylate builders encompasses the ether polycarboxylates. A number of ether polycarboxylates have been disclosed for use as detergent builders. Examples of useful ether polycarboxylates include oxydisuccinate, as disclosed in Berg, U.S. Patent 3,128,287, issued April 7, 1964, and Lamberti et al., U.S. Patent 3,635,830, issued January 18, 1972, both of which are incorporated herein by reference.

A specific type of ether polycarboxylates useful as builders in the present invention also include those having the general formula:

CH(A)(COOX)-CH(COOX)-O-CH(COOX)-CH(COOX)(B)

wherein A is H or OH; B is H or -O-CH(COOX)-CH_2(COOX); and X is H or a salt-forming cation. For example, if in the above general formula A and B are both H, then the compound is oxydissuccinic acid and its water-soluble salts. If A is OH and B is H, then the compound is tartrate monosuccinic acid (TMS) and its water-soluble salts. If A is H and B is -O-CH(COOX)-CH_2(COOX), then the compound is tartrate disuccinic acid (TDS) and its water-soluble salts. Mixtures of these builders are especially preferred for use herein. Particularly preferred are mixtures of TMS and TDS in a weight ratio of TMS to TDS of from about 97:3 to about 20:80. These builders are disclosed in U.S. Patent 4,663,071, issued to Bush et al., on May 5, 1987.

Suitable ether polycarboxylates also include cyclic compounds, particularly alicyclic compounds, such as those described in U.S. Patents 3,923,679; 3,835,163; 4,158,635; 4,120,874 and 4,102,903, all of which are incorporated herein by reference.

Other useful detergency builders include the ether

hydroxypolycarboxylates represented by th structure:

$$HO\text{-}[C(R)(COOM)\text{-}C(R)(COOM)\text{-}O]_n\text{-}H$$

wherein M is hydrogen or a cation wherein the resultant salt is water-soluble, preferably an alkali metal, ammonium or substituted ammonium cation, n is from about 2 to about 15 (preferably n is from about 2 to about 10, more preferably n averages from about 2 to about 4) and each R is the same or different and selected from hydrogen, C_{1-4} alkyl or C_{1-4} substituted alkyl (preferably R is hydrogen).

Still other ether polycarboxylates include copolymers of maleic anhydride with ethylene or vinyl methyl ether, 1, 3, 5-trihydroxy benzene-2, 4, 6-trisulphonic acid, and carboxymethyloxysuccinic acid.

Organic polycarboxylate builders also include the various alkali metal, ammonium and substituted ammonium salts of polyacetic acids. Examples include the sodium, potassium, lithium, ammonium and substituted ammonium salts of ethylenediamine tetraacetic acid, and nitrilotriacetic acid.

Also included are polycarboxylates such as mellitic acid, succinic acid, oxydisuccinic acid, polymaleic acid, benzene 1,3,5-tricarboxylic acid, and carboxymethyloxysuccinic acid, and soluble salts thereof.

Citrate builders, e.g., citric acid and soluble salts thereof (particularly sodium salt), are polycarboxylate builders of particular importance for heavy duty liquid detergent formulations, but can also be used in granular compositions.

Other carboxylate builders include the carboxylated carbohydrates disclosed in U.S. Patent 3,723,322, Diehl, issued March 28, 1973, incorporated herein by reference.

Also suitable in the detergent compositions of the present invention are the 3,3-dicarboxy-4-oxa-1,6-hexanedioates and the related compounds disclosed in U.S. Patent 4,566,984, Bush, issued January 28, 1986, incorporated herein by reference. Useful succinic acid builders include the C_5-C_{20} alkyl succinic acids and salts thereof. A particularly preferred compound of this type is dodecenylsuccinic acid. Alkyl succinic acids typically are of the general formula

$$R\text{-}CH(COOH)CH_2(COOH)$$

i.e., derivativ s of succinic acid, wherein R is hydrocarbon, e.g., C_{10}-C_{20} alkyl or alkenyl, preferably C_{12}-C_{16} or wherein R may be substitut d with hydroxyl, sulfo, sulfoxy or sulfone substituents, all as described in the above-mentioned

patents.

The succinate builders are preferably used in the form of their water-soluble salts, including the sodium, potassium, ammonium and alkanolammonium salts.

Specific examples of succinate builders include: laurylsuccinate, myristylsuccinate, palmitylsuccinate, 2-dodecenylsuccinate (preferred), 2-pentadecenylsuccinate, and the like. Laurylsuccinates are the preferred builders of this group, and are described in European Patent Application 86200690.5/0,200,263, published November 5, 1986.

Examples of useful builders also include sodium and potassium carboxymethyloxymalonate, carboxymethyloxysuccinate, cis-cyclo-hexane-hexacarboxylate, cis-cyclopentane-tetracarboxylate, water-soluble polyacrylates (these polyacrylates having molecular weights to above about 2,000 can also be effectively utilized as dispersants), and the copolymers of maleic anhydride with vinyl methyl ether or ethylene.

Other suitable polycarboxylates are the polyacetal carboxylates disclosed in U.S. Patent 4,144,226, Crutchfield et al., issued March 13, 1979, incorporated herein by reference. These polyacetal carboxylates can be prepared by bringing together, under polymerization conditions, an ester of glyoxylic acid and a polymerization initiator. The resulting polyacetal carboxylate ester is then attached to chemically stable end groups to stabilize the polyacetal carboxylate against rapid depolymerization in alkaline solution, converted to the corresponding salt, and added to a surfactant.

Polycarboxylate builders are also disclosed in U.S. Patent 3,308,067, Diehl, issued March 7, 1967, incorporated herein by reference. Such materials include the water-soluble salts of homo- and copolymers of aliphatic carboxylic acids such as maleic acid, itaconic acid and methylenemalonic acid.

Other organic builders known in the art can also be used. For example, monocarboxylic acids, and soluble salts thereof, having long chain hydrocarbyls can be utilized. These would include materials generally referred to as "soaps." Chain lengths of C_{10}-C_{20} are typically utilized. The hydrocarbyls can be saturated or unsaturated.

Other optional ingredients include chelating agents, clay soil removal/anti redeposition agents, polymeric dispersing agents, bleaches, brighteners, suds suppressors, solvents and aesthetic ag nts.

VI. Working xamples of compositions

The detergent composition herein can be formulated as a variety of compositions, for instance as laundry detergents as well as hard surface cleaners or dishwashing compositions.

1. Hard surface cleaning compositions

As used herein "hard surface cleaning composition" refers to liquid and granular detergent compositions for cleaning hard surfaces such as floors, walls, bathroom tile, and the like. Hard surface cleaning compositions of the present invention comprise an effective amount of one or more fusion proteins of the present invention, preferably from about 0.001% to about 10%, more preferably from about .01% to about 5%, more preferably still from about .05% to about 1% by weight of active of the composition. In addition to comprising one or more fusion proteins, such hard surface cleaning compositions typically comprise a surfactant and a water-soluble sequestering builder. In certain specialized products such as spray window cleaners, however, the surfactants are sometimes not used since they may produce a filmy/streaky residue on the glass surface.

The surfactant component, when present, may comprise as little as 0.1% of the compositions herein, but typically the compositions will contain from about 0.25% to about 10%, more preferably from about 1% to about 5% of surfactant.

Typically the compositions will contain from about 0.5% to about 50% of a detergency builder, preferably from about 1% to about 10%.

Preferably the pH should be in the range of about 8 to 12. Conventional pH adjustment agents such as sodium hydroxide, sodium carbonate or hydrochloric acid can be used if adjustment is necessary.

Solvents may be included in the compositions. Useful solvents include, but are not limited to, glycol ethers such as diethyleneglycol monohexyl ether, diethyleneglycol monobutyl ether, ethyleneglycol monobutyl ether, ethyleneglycol monohexyl ether, propyleneglycol monobutyl ether, dipropyleneglycol monobutyl ether, and diols such as 2,2,4-trimethyl-1,3-pentanediol and 2-ethyl-1,3-hexanediol. When used, such solvents are typically present at levels of from about 0.5% to about 15%, preferably from about 3% to about 11%.

Additionally, highly volatile solvents such as isopropanol or ethanol can be used in the present compositions to facilitate faster evaporation of the composition from surfaces wh n the surface is not rinsed after "full strength" application of the composition to the surface. When used, volatile solvents are

typically present at l vels of from about 2% to about 12% in th compositions.

The hard surface cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 7-12

Liquid Hard Surface Cleaning Compositions

	Example No.					
Component	7	8	9	10	11	12
Fusion protein of Ex. 7	0.05	0.50	0.02	0.03	0.10	0.03
Fusion protein of Ex. 10	–	–	-	-	0.20	0.02
Na_2DIDA*						
EDTA**	–	–	2.90	2.90	–	–
Na Citrate	–	–	–	–	2.90	2.90
NaC_{12} Alkyl-benzene sulfonate	1.95	–	1.95	–	1.95	–
NaC_{12} Alkylsulfate	–	2.20	–	2.20	–	2.20
NaC_{12}(ethoxy)*** sulfate	–	2.20	–	2.20	–	2.20
C_{12} Dimethylamine oxide	–	0.50	–	0.50	–	0.50
Na Cumene sulfonate	1.30	–	1.30	–	1.30	–
Hexyl Carbitol***	6.30	6.30	6.30	6.30	6.30	6.30
Water****	balance to 100%					

*Disodium N-diethyleneglycol-N,N-iminodiacetate

**Na_4 ethylenediamine diacetic acid

***Diethyleneglycol monohexyl ether

****All formulas adjusted to pH 7

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Examples 13-18
Spray Compositions for Cleaning Hard Surfaces
and Removing Household Mildew

	Example No.					
Component	13	14	15	16	17	18
Fusion protein of Ex. 7	0.50	0.05	0.60	0.30	0.20	0.30
Fusion protein of Ex. 11	-	-	-	-	0.30	0.10
Sodium octyl sulfate	2.00	2.00	2.00	2.00	2.00	2.00
Sodium dodecyl sulfate	4.00	4.00	4.00	4.00	4.00	4.00
Sodium hydroxide	0.80	0.80	0.80	0.80	0.80	0.80
Silicate (Na)	0.04	0.04	0.04	0.04	0.04	0.04
Perfume	0.35	0.35	0.35	0.35	0.35	0.35
Water	balance to 100%					

Product pH is about 7.

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

2. Dishwashing Compositions

In another embodiment of the present invention, dishwashing compositions comprise one or more fusion proteins of the present invention. As used herein, "dishwashing composition" refers to all forms for compositions for cleaning dishes, including but not limited to, granular and liquid forms. The dishwashing composition embodiment of the present invention is illustrated by the following examples.

Examples 19-24

Dishwashing Composition

Component	Example No. 19	20	21	22	23	24
Fusion protein of Ex. 7	0.05	0.50	0.02	0.40	0.10	0.03
Fusion protein of Ex. 10	-	-	-	-	0.40	0.02
C_{12}-C_{14} N-methyl-glucamide	0.90	0.90	0.90	0.90	0.90	0.90
C_{12} ethoxy (1) sulfate	12.00	12.00	12.00	12.00	12.00	12.00
2-methyl undecanoic acid	4.50	4.50	4.50	4.50	4.50	4.50
C_{12} ethoxy (2) carboxylate	4.50	4.50	4.50	4.50	4.50	4.50
C_{12} alcohol ethoxylate (4)	3.00	3.00	3.00	3.00	3.00	3.00
C_{12} amine oxide	3.00	3.00	3.00	3.00	3.00	3.00
Sodium cumene sulfonate	2.00	2.00	2.00	2.00	2.00	2.00
Ethanol	4.00	4.00	4.00	4.00	4.00	4.00
Mg^{++} (as $MgCl_2$)	0.20	0.20	0.20	0.20	0.20	0.20
Ca^{++} (as $CaCl_2$)	0.40	0.40	0.40	0.40	0.40	0.40
Water	balance to 100%					

Product pH is adjusted to 7.

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

3. Fabric cleaning compositions

In another embodiment of the present invention, fabric cleaning compositions comprise one or more fusion proteins of the present invention. As used herein, "fabric cleaning composition" refers to all forms for detergent compositions for cleaning fabrics, including but not limited to, granular, liquid and bar forms. Preferred fabric cleaning compositions are those in the liquid form.

a. Granular fabric cleaning compositions

The granular fabric cleaning compositions of the present invention contain an effective amount of one or more fusion proteins of the present inv ntion, preferably from about 0.001% to about 10%, more preferably from about 0.005% to about 5%, more pref rably from about 0.01% to about 1% by weight of activ of the composition. In addition to one or more fusion prot ins,

th granular fabric cleaning compositions typically comprise at least on surfactant, one or more builders, and, in some cases, a bleaching agent.

The granular fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 25-28

Granular Fabric Cleaning Composition

	Example No.			
Component	25	26	27	28
Fusion protein of Ex. 7	0.10	0.20	0.03	0.05
Fusion protein of Ex. 11	-	-	0.02	0.05
C_{13} linear alkyl benzene sulfonate	22.00	22.00	22.00	22.00
Phosphate (as sodium tripolyphosphates)	23.00	23.00	23.00	23.00
Sodium carbonate	23.00	23.00	23.00	23.00
Sodium silicate	14.00	14.00	14.00	14.00
Zeolite	8.20	8.20	8.20	8.20
Chelant (diethylaenetriamine-pentaacetic acid)	0.40	0.40	0.40	0.40
Sodium sulfate	5.50	5.50	5.50	5.50
Water	balance to 100%			

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Examples 29-32

Granular Fabric Cleaning Composition

Component	Example No. 29	30	31	32
Fusion protein of Ex. 7	0.10	0.20	0.03	0.05
Fusion protein of Ex. 10	-	-	0.02	0.05
C_{12} alkyl benzene sulfonate	12.00	12.00	12.00	12.00
Zeolite A (1-10 micrometer)	26.00	26.00	26.00	26.00
2-butyl octanoic acid	4.00	4.00	4.00	4.00
C_{12}-C_{14} secondary (2,3) alkyl sulfate, Na salt	5.00	5.00	5.00	5.00
Sodium citrate	5.00	5.00	5.00	5.00
Optical brightener	0.10	0.10	0.10	0.10
Sodium sulfate	17.00	17.00	17.00	17.00
Water and minors	balance to 100%			

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Examples 33-36

Granular Fabric Cleaning Composition

Component	Example No. 33	34	35	36
Fusion protein of Ex. 7	0.10	0.20	0.03	0.05
Fusion protein of Ex. 11	-	-	0.02	0.05
C_{13} linear alkyl benzene sulfonate	22.00	22.00	22.00	22.00
Phosphate (as sodium tripolyphosphates)	23.00	23.00	23.00	23.00
Sodium carbonate	23.00	23.00	23.00	23.00
Sodium silicate	14.00	14.00	14.00	14.00
Zeolite	8.20	8.20	8.20	8.20
Chelant (diethylaenetriamine-pentaacetic acid)	0.40	0.40	0.40	0.40
Sodium sulfate	5.50	5.50	5.50	5.50
Water	balance to 100%			

In th examples above any of the fusion prot ins recited or claim d are substituted for th fusion prot in(s) shown abov , with substantially similar

results.

Examples 37-40

Granular Fabric Cleaning Composition

	Example No.			
Component	37	38	39	40
Fusion protein of Ex. 7	0.10	0.20	0.03	0.05
Fusion protein of Ex. 10	-	-	0.02	0.05
C_{12} alkyl benzene sulfonate	12.00	12.00	12.00	12.00
Zeolite A (1-10 micrometer)	26.00	26.00	26.00	26.00
2-butyl octanoic acid	4.00	4.00	4.00	4.00
C_{12}-C_{14} secondary (2,3) alkyl sulfate, Na salt	5.00	5.00	5.00	5.00
Sodium citrate	5.00	5.00	5.00	5.00
Optical brightener	0.10	0.10	0.10	0.10
Sodium sulfate	17.00	17.00	17.00	17.00
Water and minors	balance to 100%			

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Examples 41-42

Granular Fabric Cleaning Composition

Component	Example No. 41	42
Linear alkyl benzene sulphonate	11.4	10.70
Tallow alkyl sulphate	1.80	2.40
C_{14-15} alkyl sulphate	3.00	3.10
C_{14-15} alcohol 7 times ethoxylated	4.00	4.00
Tallow alcohol 11 times ethoxylated	1.80	1.80
Dispersant	0.07	0.1
Silicone fluid	0.80	0.80
Trisodium citrate	14.00	15.00
Citric acid	3.00	2.50
Zeolite	32.50	32.10
Maleic acid acrylic acid copolymer	5.00	5.00
Diethylene triamine penta methylene phosphonic acid	1.00	0.20
Fusion protein of Ex. 7	0.30	0.30
Lipase	0.36	0.40
Amylase	0.30	0.30
Sodium silicate	2.00	2.50
Sodium sulphate	3.50	5.20
Polyvinyl pyrrolidone	0.30	0.50
Perborate	0.5	1
Phenol sulphonate	0.1	0.2
Peroxidase	0.1	0.1
Minors	Up to 100	Up to 100

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Exampl s 43-44

Granular Fabric Cleaning Composition

	Example No.	
Component	43	44
Sodium linear C_{12} alkyl benzene-sulfonate	6.5	8.0
Sodium sulfate	15.0	18.0
Zeolite A	26.0	22.0
Sodium nitrilotriacetate	5.0	5.0
Polyvinyl pyrrolidone	0.5	0.7
Tetraacetylethylene diamine	3.0	3.0
Boric acid	4.0	-
Perborate	0.5	1
Phenol sulphonate	0.1	0.2
Fusion protein of Ex. 11	0.4	0.4
Fillers (e.g., silicates; carbonates; perfumes; water)	Up to 100	Up to 100

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Exampl 45

Compact Granular Fabric Cleaning Composition

Component	Weight %
Alkyl Sulphate	8.0
Alkyl Ethoxy Sulphate	2.0
Mix of C_{25} and C_{45} alcohol 3 and 7 times ethoxylated	6.0
Polyhydroxy fatty acid amide	2.5
Zeolite	17.0
Layered silicate/citrate	16.0
Carbonate	7.0
Maleic acid acrylic acid copolymer	5.0
Soil release polymer	0.4
Carboxymethyl cellulose	0.4
Poly (4-vinylpyridine) -N-oxide	0.1
Copolymer of vinylimidazole and vinylpyrrolidone	0.1
PEG2000	0.2
Pro209Gln	0.5
Fusion protein of Ex. 7	0.2
Cellulase	0.2
Tetracetylethylene diamine	6.0
Percarbonate	22.0
Ethylene diamine disuccinic acid	0.3
Suds suppressor	3.5
Disodium-4,4'-bis (2-morpholino -4-anilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate	0.25
Disodium-4,4'-bis (2-sulfostyril) biphenyl	0.05
Water, Perfume and Minors	Up to 100

In the example above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Example 46

Granular Fabric Cleaning Composition

Component	Weight %
Linear alkyl benzene sulphonate	7.6
C_{16}-C_{18} alkyl sulfate	1.3
C_{14-15} alcohol 7 times ethoxylated	4.0
Coco-alkyl-dimethyl hydroxyethyl ammonium chloride	1.4
Dispersant	0.07
Silicone fluid	0.8
Trisodium citrate	5.0
Zeolite 4A	15.0
Maleic acid acrylic acid copolymer	4.0
Diethylene triamine penta methylene phosphonic acid	0.4
Perborate	15.0
Tetraacetylethylene diamine	5.0
Smectite clay	10.0
Poly (oxy ethylene) (MW 300,000)	0.3
Fusion protein of Ex. 7	0.4
Lipase	0.2
Amylase	0.3
Cellulase	0.2
Sodium silicate	3.0
Sodium carbonate	10.0
Carboxymethyl cellulose	0.2
Brighteners	0.2
Water, perfume and minors	Up to 100

In the example above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Example 47

Granular Fabric Cleaning Composition

Component	Weight %
Linear alkyl benzene sulfonate	6.92
Tallow alkyl sulfate	2.05
C_{14-15} alcohol 7 times ethoxylated	4.4
C_{12-15} alkyl ethoxy sulfate - 3 times ethoxylated	0.16
Zeolite	20.2
Citrate	5.5
Carbonate	15.4
Silicate	3.0
Maleic acid acrylic acid copolymer	4.0
Carboxymethyl cellulase	0.31
Soil release polymer	0.30
Fusion protein of Ex. 7	0.2
Lipase	0.36
Cellulase	0.13
Perborate tetrahydrate	11.64
Perborate monohydrate	8.7
Tetraacetylethylene diamine	5.0
Diethylene tramine penta methyl phosphonic acid	0.38
Magnesium sulfate	0.40
Brightener	0.19
Perfume, silicone, suds suppressors	0.85
Minors	Up to 100

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

b. Liquid fabric cleaning compositions

Liquid fabric cleaning compositions of the present invention comprise an effective amount of one or more fusion proteins of the present invention, preferably from about 0.005% to about 5%, more preferably from about 0.01% to about 1%, by weight of active of the composition. Such liquid fabric cleaning compositions typically additionally comprise an anionic surfactant, a fatty acid, a wat r-soluble d tergency builder and water.

Th liquid fabric cleaning composition embodiment of the present

inv ntion is illustrated by th following exampl s.

Examples 48-52

Liquid Fabric Cleaning Compositions

	Example No.				
Component	48	49	50	51	52
Fusion protein of Ex. 7	0.05	0.03	0.30	0.03	0.10
Fusion protein of Ex. 10	-	-	-	0.01	0.20
C_{12}- C_{14} alkyl sulfate, Na	20.00	20.00	20.00	20.00	20.00
2-butyl octanoic acid	5.00	5.00	5.00	5.00	5.00
Sodium citrate	1.00	1.00	1.00	1.00	1.00
C_{10} alcohol ethoxylate (3)	13.00	13.00	13.00	13.00	13.00
Monethanolamine	2.50	2.50	2.50	2.50	2.50
Water/propylene glycol/ethanol (100:1:1)	balance to 100%				

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Examples 53-57

Liquid Fabric Cleaning Compositions

	Example No.				
Component	53	54	55	56	57
Fusion protein of Ex. 7	0.05	0.03	0.30	0.03	0.10
Fusion protein of Ex. 10	–	–	–	0.01	0.20
C_{12}- C_{14} alkyl sulfate, Na	20.00	20.00	20.00	20.00	20.00
2-butyl octanoic acid	5.00	5.00	5.00	5.00	5.00
Sodium citrate	1.00	1.00	1.00	1.00	1.00
C_{10} alcohol ethoxylate (3)	13.00	13.00	13.00	13.00	13.00
Monethanolamine	2.50	2.50	2.50	2.50	2.50
Water/propylene glycol/ethanol (100:1:1)	balance to 100%				

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Examples 58-59

Granular Fabric Cleaning Composition

Component	Example No. 58	59
C_{12-14} alkenyl succinic acid	3.0	8.0
Citric acid monohydrate	10.0	15.0
Sodium C_{12-15} alkyl sulphate	8.0	8.0
Sodium sulfate of C_{12-15} alcohol 2 times ethoxylated	-	3.0
C_{12-15} alcohol 7 times ethoxylated	-	8.0
C_{12-15} alcohol 5 times ethoxylated	8.0	-
Diethylene triamine penta (methylene phosphonic acid)	0.2	-
Oleic acid	1.8	-
Ethanol	4.0	4.0
Propanediol	2.0	2.0
Fusion protein of Ex. 7	0.2	0.2
Polyvinyl pyrrolidone	1.0	2.0
Suds suppressor	0.15	0.15
NaOH	up to pH 7.5	
Perborate	0.5	1
Phenol sulphonate	0.1	0.2
Peroxidase	0.4	0.1
Waters and minors	up to 100 parts	

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Exampl s 60-62

Liquid Fabric Cleaning Composition

	Example No.		
Component	60	61	62
Citric Acid	7.10	3.00	3.00
Fatty Acid	2.00	-	2.00
Ethanol	1.93	3.20	3.20
Boric Acid	2.22	3.50	3.50
Monoethanolamine	0.71	1.09	1.09
1,2 Propanediol	7.89	8.00	8.00
NaCumene Sulfonate	1.80	3.00	3.00
NaFormate	0.08	0.08	0.08
NaOH	6.70	3.80	3.80
Silicon anti-foam agent	1.16	1.18	1.18
Fusion protein of Ex. 7	0.0145	-	-
Fusion protein of Ex. 10	-	0.0145	-
Fusion protein of Ex. 11	-	-	0.0145
Lipase	0.200	0.200	0.200
Cellulase	-	7.50	7.50
Soil release polymer	0.29	0.15	0.15
Anti-foaming agents	0.06	0.085	0.085
Brightener 36	0.095	-	-
Brightener 3	-	0.05	0.05
C_{12} alkyl benzenesulfonic acid	9.86	-	-
C_{12-15} alkyl polyethoxylate (2.5) sulfate	13.80	18.00	18.00
C_{12} glucose amide	-	5.00	5.00
C_{12-13} alkyl polyethoxylate (9)	2.00	2.00	2.00
Water, perfume and minors	balance to 100%		

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

c. Bar fabric cleaning compositions

Bar fabric cleaning compositions of the present invention suitable for hand-washing soiled fabrics contain an effectiv amount of on or more fusion proteins of the pres nt invention, pref rably from about 0.001% to about 10%, more preferably from about 0.01% to about 1% by weight of the composition.

The bar fabric cleaning composition embodiment of th present invention is illustrated by the following examples.

Examples 63-66

Bar Fabric Cleaning Compositions

	Example No.			
Component	63	64	65	66
Fusion protein of Ex. 7	0.3	-	0.1	0.02
Fusion protein of Ex. 10	-	-	0.4	0.03
C_{12}-C_{16} alkyl sulfate, Na	20.0	20.0	20.0	20.00
C_{12}-C_{14} N-methyl glucamide	5.0	5.0	5.0	5.00
C_{11}-C_{13} alkyl benzene sulfonate, Na	10.0	10.0	10.0	10.00
Sodium carbonate	25.0	25.0	25.0	25.00
Sodium pyrophosphate	7.0	7.0	7.0	7.00
Sodium tripolyphosphate	7.0	7.0	7.0	7.00
Zeolite A (0.1-.10)	5.0	5.0	5.0	5.00
Carboxymethylcellulose	0.2	0.2	0.2	0.20
Polyacrylate (MW 1400)	0.2	0.2	0.2	0.20
Coconut monethanolamide	5.0	5.0	5.0	5.00
Brightener, perfume	0.2	0.2	0.2	0.20
$CaSO_4$	1.0	1.0	1.0	1.00
$MgSO_4$	1.0	1.0	1.0	1.00
Water	4.0	4.0	4.0	4.00
Filler*	balance to 100%			

*Can be selected from convenient materials such as $CaCO_3$, talc, clay, silicates, and the like.

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Exampl s 67-70

Bar Fabric Cleaning Compositions

	Example No.			
Component	67	68	69	70
Fusion protein of Ex. 7	0.3	-	0.1	0.02
Fusion protein of Ex. 11	-	0.3	0.4	0.03
C_{12}-C_{16} alkyl sulfate, Na	20.0	20.0	20.0	20.00
C_{12}-C_{14} N-methyl glucamide	5.0	5.0	5.0	5.00
C_{11}-C_{13} alkyl benzene sulfonate, Na	10.0	10.0	10.0	10.00
Sodium carbonate	25.0	25.0	25.0	25.00
Sodium pyrophosphate	7.0	7.0	7.0	7.00
Sodium tripolyphosphate	7.0	7.0	7.0	7.00
Zeolite A (0.1-.10)	5.0	5.0	5.0	5.00
Carboxymethylcellulose	0.2	0.2	0.2	0.20
Polyacrylate (MW 1400)	0.2	0.2	0.2	0.20
Coconut monethanolamide	5.0	5.0	5.0	5.00
Brightener, perfume	0.2	0.2	0.2	0.20
$CaSO_4$	1.0	1.0	1.0	1.00
$MgSO_4$	1.0	1.0	1.0	1.00
Water	4.0	4.0	4.0	4.00
Filler*	balance to 100%			

*Can be selected from convenient materials such as $CaCO_3$, talc, clay, silicates, and the like.

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

B. Additional Cleaning Compositions

In addition to the hard surface cleaning, dishwashing and fabric cleaning compositions discussed above, one or more fusion proteins of the present invention may be incorporated into a variety of other cleaning compositions where hydrolysis of an insoluble substrate is desired. Such additional cleaning compositions include but are not limited to, oral cleaning compositions, denture cleaning compositions, and contact lens cleaning compositions.

1. Oral cleaning compositions

In anoth r mbodiment of the present inv ntion, a pharmaceutically-acceptable amount of on or more fusion proteins of the present inv ntion are

included in compositions us ful for removing peptide stains from teeth or dentures. As used herein, "oral cleaning compositions" refers to dentifrices, toothpastes, toothgels, toothpowders, mouthwashes, mouth sprays, mouth gels, chewing gums, lozenges, sachets, tablets, biogels, prophylaxis pastes, dental treatment solutions, and the like. Preferably, the oral cleaning compositions comprise from about 0.0001% to about 20% of one or more fusion proteins of the present invention, more preferably from about 0.001% to about 10%, more preferably still from about 0.01% to about 5%, by weight of the composition, and a pharmaceutically-acceptable carrier. As used herein, "pharmaceutically-acceptable" means that drugs, medicaments or inert ingredients which the term describes are suitable for use in contact with the tissues of humans and lower animals without undue toxicity, incompatibility, instability, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio.

Typically, the pharmaceutically-acceptable oral cleaning carrier components of the oral cleaning components of the oral cleaning compositions will generally comprise from about 50% to about 99.99%, preferably from about 65% to about 99.99%, more preferably from about 65% to about 99%, by weight of the composition.

The pharmaceutically-acceptable carrier components and optional components which may be included in the oral cleaning compositions of the present invention are well known to those skilled in the art. A wide variety of composition types, carrier components and optional components useful in the oral cleaning compositions are disclosed in U.S. Patent 5,096,700, Seibel, issued March 17, 1992; U.S. Patent 5,028,414, Sampathkumar, issued July 2, 1991; and U.S. Patent 5,028,415, Benedict, Bush and Sunberg, issued July 2, 1991; all of which are incorporated herein by reference.

The oral cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 71-74

Dentifrice Composition

Component	Example No. 71	72	73	74
Fusion protein of Ex. 7	2.000	3.500	1.500	2.000
Sorbitol (70% aqueous solution)	35.000	35.000	35.000	35.000
PEG-6*	1.000	1.000	1.000	1.000
Silica dental abrasive**	20.000	20.000	20.000	20.000
Sodium fluoride	0.243	0.243	0.243	0.243
Titanium dioxide	0.500	0.500	0.500	0.500
Sodium saccharin	0.286	0.286	0.286	0.286
Sodium alkyl sulfate (27.9% aqueous solution)	4.000	4.000	4.000	4.000
Flavor	1.040	1.040	1.040	1.040
Carboxyvinyl Polymer***	0.300	0.300	0.300	0.300
Carrageenan****	0.800	0.800	0.800	0.800
Water	balance to 100%			

*PEG-6 = Polyethylene glycol having a molecular weight of 600.

**Precipitated silica identified as Zeodent 119 offered by J.M. Huber.

***Carbopol offered by B.F. Goodrich Chemical Company.

****Iota Carrageenan offered by Hercules Chemical Company.

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Examples 75-78

Mouthwash Composition

	Example No.			
Component	75	76	77	78
Fusion protein of Ex. 7	3.00	7.50	1.00	5.00
SDA 40 Alcohol	8.00	8.00	8.00	8.00
Flavor	0.08	0.08	0.08	0.08
Emulsifier	0.08	0.08	0.08	0.08
Sodium Fluoride	0.05	0.05	0.05	0.05
Glycerin	10.00	10.00	10.00	10.00
Sweetener	0.02	0.02	0.02	0.02
Benzoic acid	0.05	0.05	0.05	0.05
Sodium hydroxide	0.20	0.20	0.20	0.20
Dye	0.04	0.04	0.04	0.04
Water	balance to 100%			

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Examples 79-82

Lozenge Composition

	Example No.			
Component	79	80	81	82
Fusion protein of Ex. 7	0.01	0.03	0.10	0.02
Sorbitol	17.50	17.50	17.50	17.50
Mannitol	17.50	17.50	17.50	17.50
Starch	13.60	13.60	13.60	13.60
Sweetener	1.20	1.20	1.20	1.20
Flavor	11.70	11.70	11.70	11.70
Color	0.10	0.10	0.10	0.10
Corn Syrup	balance to 100%			

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Examples 83-86

Chewing Gum Composition

	Example No.			
Component	83	84	85	86
Fusion protein of Ex. 7	0.03	0.02	0.10	0.05
Sorbitol crystals	38.44	38.40	38.40	38.40
Paloja-T gum base*	20.00	20.00	20.00	20.00
Sorbitol (70% aqueous solution)	22.00	22.00	22.00	22.00
Mannitol	10.00	10.00	10.00	10.00
Glycerine	7.56	7.56	7.56	7.56
Flavor	1.00	1.00	1.00	1.00

*Supplied by L.A. Dreyfus Company.

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

2. Denture cleaning compositions

In another embodiment of the present invention, denture cleaning compositions for cleaning dentures outside of the oral cavity comprise one or more fusion proteins of the present invention. Such denture cleaning compositions comprise an effective amount of one or more fusion proteins, preferably from about 0.0001% to about 50% of one or more fusion proteins, more preferably from about 0.001% to about 35%, more preferably still from about 0.01% to about 20%, by weight of the composition, and a denture cleansing carrier. Various denture cleansing composition formats such as effervescent tablets and the like are well known in the art (see for example U.S. Patent 5,055,305, Young, incorporated herein by reference), and are generally appropriate for incorporation of one or more fusion proteins for removing peptide stains from dentures.

The denture cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 87-90

Two-layer Effervescent Denture Cleansing Tablet

Component	Example No. 87	88	89	90
Acidic Layer				
Fusion protein of Ex. 10	1.0	1.5	0.01	0.05
Tartaric acid	24.0	24.0	24.00	24.00
Sodium carbonate	4.0	4.0	4.00	4.00
Sulphamic acid	10.0	10.0	10.00	10.00
PEG 20,000	4.0	4.0	4.00	4.00
Sodium bicarbonate	24.5	24.5	24.50	24.50
Potassium persulfate	15.0	15.0	15.00	15.00
Sodium acid pyrophosphate	7.0	7.0	7.00	7.00
Pyrogenic silica	2.0	2.0	2.00	2.00
TAED*	7.0	7.0	7.00	7.00
Ricinoleylsulfosuccinate	0.5	0.5	0.50	0.50
Flavor	1.0	1.0	1.00	1.00
Alkaline Layer				
Sodium perborate monohydrate	32.0	32.0	32.00	32.00
Sodium bicarbonate	19.0	19.0	19.00	19.00
EDTA	3.0	3.0	3.00	3.00
Sodium tripolyphosphate	12.0	12.0	12.00	12.00
PEG 20,000	2.0	2.0	2.00	2.00
Potassium persulfate	26.0	26.0	26.00	26.00
Sodium carbonate	2.0	2.0	2.00	2.00
Pyrogenic silica	2.0	2.0	2.00	2.00
Dye/flavor	2.0	2.0	2.00	2.00

*Tetraacetylethylene diamine

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

3. Contact Lens Cleaning Compositions

In another embodiment of the present invention, contact lens cleaning compositions comprise one or more fusion proteins of the present invention. Such contact lens cleaning compositions comprise an effective amount of one or

more fusion proteins, pref rably from about 0.01% to about 50% of one or more fusion proteins, more preferably from about 0.01% to about 20%, more preferably still from about 1% to about 5%, by weight of the composition, and a contact lens cleaning carrier. Various contact lens cleaning composition formats such as tablets, liquids and the like are well known in the art (see for example U.S. Patent 4,863,627, Davies, Meaken and Rees, issued September 5, 1989; U.S. Patent Re. 32,672, Huth, Lam and Kirai, reissued May 24, 1988; U.S. Patent 4,609,493, Schäfer, issued September 2, 1986; U.S. Patent, 4,690,793, Ogunbiyi and Smith, issued September 1, 1987; U.S. Patent 4,614,549, Ogunbiyi, Riedhammer and Smith, issued September 30, 1986; and U.S. Patent 4,285,738, Ogata, issued August 25, 1981; each of which are incorporated herein by reference), and are generally appropriate for incorporation of one or more fusion proteins of the present invention for removing peptide stains from contact lens.

The contact lens cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 91-94

Enzymatic Contact Lens Cleaning Solution

	Example No.			
Component	91	92	93	94
Fusion protein of Ex. 10	0.01	0.5	0.1	2.0
Glucose	50.00	50.0	50.0	50.0
Nonionic surfactant (polyoxyethlene-polyoxypropylene copolymer)	2.00	2.0	2.0	2.0
Anionic surfactant (polyoxyethylene-alkylphenylether sodium sulfricester)	1.00	1.0	1.0	1.0
Sodium chloride	1.00	1.0	1.0	1.0
Borax	0.30	0.3	0.3	0.3
Water	balance to 100%			

In the examples above any of the fusion proteins recited or claimed are substituted for the fusion protein(s) shown above, with substantially similar results.

Example 95

An inhibitor having a K_i value of 1×10^{-8}, prepared as in Example 7, is used in a liquid cleaning composition according to example 61. Characterization of the composition shows that the composition has more than 85% of the

proteas activity aft r 1 month.

Upon use in a standard washing machine using a normal load and warm water it displays improved stain removal compared to the similar composition which differs only in using the wild type inhibitor and the standard protease.

All references cited in this application are hereby incorporated into it by reference.

As is recognized in the art, there are occasionally errors in DNA and amino acid sequencing methods. As a result, the sequences encoded in the deposited material are incorporated herein by reference and controlling in the event of an error in any of the sequences found in the written description of the present invention. It is further noted that one of ordinary skill in the art reproducing Applicants' work from the written disclosure can discover any sequencing errors using routine skill. The deposit of ATCC No. 69954, 69955, and 98025 are not to be considered as an admission that the deposited material is essential to the practice of the present invention.

While particular embodiments of the subject invention have been described, it will be obvious to those skilled in the art that various changes and modifications of the subject invention can be made without departing from the spirit and scope of the invention. It is intended to cover, in the appended claims, all such modifications that are within the scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SAUNDERS, CHARLES W.

(ii) TITLE OF INVENTION: PROTEASES AND THEIR VARIANTS HAVING PEPTIDE PROTEASE INHIBITORS FUSED TO THEM

(iii) NUMBER OF SEQUENCES: 23

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: THE PROCTER & GAMBLE COMPANY
(B) STREET: 11810 EAST MIAMI RIVER ROAD
(C) CITY: ROSS
(D) STATE: OH
(E) COUNTRY: USA
(F) ZIP: 45061

(v) COMPUTER READABLE FORM:
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(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: HAKE, RICHARD A.
(B) REGISTRATION NUMBER: 37,343
(C) REFERENCE/DOCKET NUMBER: FUS

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: 513-627-0260

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAAACAACTT TCAACAGTCT CGAGACTAGT TTCAGCGGAG TGAGAATA
48

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TACTTCACTC TGCATTACCC GCAGTACGAC GTTTACTTCC TGCCGGAAGG TTCTCCTGTT
60

ACTCTGGACC TGCGTTAC
78

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 75 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAGAGTAACA GGAGAACCTT CCGGCAGGAA GTAAACGTCG TACTGCGGGT AATGCAGAGT
60

GAAGTATTCA CGAGC
75

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATTCACTGA ATTTGGTTCT GAACTGAAAT CTTTCCCAGA AGTTGTTGGT AAAACTGTTG
60

ACCAGGCTCG TGAA
74

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTGTAGAAA ACACGAACAC GGTTGTAACG CAGGTC
36

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATTACGAAT TCACTGAATT TGGTTCTGAA
30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCTAGAGGAT CCTAACCAAC ATGCGGA
27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GATTACGAAT TCACTGAATT TGGTTCTGAA CTGAAATCTT TCCCAGAAGT TGTTGGTAAA
60

ACTGTTGACC AGGCTCGTGA A
81

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCTAGAGGAT CCTAACCAAC ATGCGGAACA TGGTTAACAA CGTTAGTACC TGGGTTGTAG
60

AAAACACGAA CACGGTTGTA ACGCAGGTC
89

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCCGACGAAT TCGATGCTCC TTCTGCACTT TATGCACCTT CAGCATTAGT TTTAACAGTT
60

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCTGAAAGAG CAGTAACTCT TACATGTGCT CCAGGCCCTT CTGGTACACA TCCAGCAGCT
60

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCTCCTACT GCAGCTAAAT CTGCACATGC AGAGCCAGCT GCTGGATGTG T
51

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TACTGCTCTT TCAGGTGCAG CTGTCGTAGC GCTAACTCCT TTACCAACTG TTAAAACTAA
60

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GATTTAGCTG CAGTAGGAGG TGACTTAAAC GCATTAACAC GTGGTGAAGA CGTTATGTGT
60

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTTGATGGAG TTTGGCAAGG TAAACGCGTA TCTTATGAAC GTGTATTTTC AAATGAATGT
60

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGTCCAAAGC TTGGATCCTT AAAATGCAAA TACAGAAGAG CCATGAGCGT TCATTTCACA
60

TTCATTTGAA AA
72

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCAAACTCCA TCAACAGTCA GTAATACAGG ATCATAAACC ATTGGACACA TAACGTCTTC
60

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGATCCAAGC TTTTCCGCAA TTATATCATT
30

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGATTCAAGC TTTGCTCAGT TTTGCTTCTG
30

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAGCTTCAGG ATGTTCATAA TTTTTAAAGA
30

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCCCAGGCGG CAGGGGAATT CAAATCAAAC GGGGAA
36

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCGGCAGCTC AGTAAGGATC CAACATAAAA AACCGGC
37

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTACAGGCGG CAGCTCAGGA ATTCTAGTAA GGATCCAACA TA
42

WHAT IS CLAIMED IS:

1. A detergent compatible fusion protein comprising a detergent compatible protease part and a protein protease inhibitor part, wherein the protease and inhibitor are linked together by a peptide chain.

2. A protease inhibitor variant according to any preceding claim wherein the peptide inhibitor part is SSI or a variant of SSI.

3. The fusion protein of any preceding claim wherein the protease part is an alkaline protease.

4. The fusion protein of any preceding claim wherein the inhibitors part of the fusion protein has a Ki such that the protease part is inhibited in the composition and provides free protease during the cleaning process.

5. A fusion protein according to any preceding claim in dry or concentrated liquid form.

6. A cleaning composition comprising one or more fusion proteins according to any preceding claim.

7. DNA encoding the fusion protein of claims 1-6.

8. An expression system comprising the DNA of claim 7 wherein the system is a living organism comprising a plasmid encoding the fusion protein.

9. A test kit comprising the fusion protein of Claim 1 or an antibody to the fusion protein.

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 97/16354

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/64 C07K14/81

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 319 944 A (ZYMOGENETICS INC) 14 June 1989 see page 2, line 46-51 see page 3, line 6-8 see page 7, line 31-33 ---	1-9
Y	S.TAGUCHI ET AL.: "Microbial secretion of biologically active human transforming growth factor alpha fused to the Streptomyces protease inhibior" GENE, vol. 159, 1995, XP002052073 see abstract, Fig.1 and 2 ---	1-9
A	WO 93 20175 A (NOVONORDISK AS ;HALKIER TORBEN (DK); CLAUSEN IB GROTH (DK); NIELSE) 14 October 1993 --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
14 January 1998	30. 01. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Deffner, C-A

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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 97/16354

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 13125 A (NOVONORDISK AS) 8 July 1993	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna..nal Application No
PCT/US 97/16354

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